Aus dem Frauen-, Kinder- und Perinatalzentrum
Campus Benjamin Franklin
der Medizinischen Fakultät der Charité – Universitätsmedizin Berlin

DISSERTATION

TRANSCRIPTIONAL REGULATION OF 12/15-LIPOXYGENASE EXPRESSION AND THE IMPLICATION OF THE ENZYME IN HEPOXILIN BIOSYNTHESIS AND APOPTOSIS

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von

Shankaranarayanan Pattabhiraman
aus Rasipuram, INDIA

Dekane: Prof. Dr. Joachim W. Dudenhausen
Prof. Dr. med. Martin Paul

Gutachter: 1. Prof. Dr. Hartmut Kühn
2. Prof. Dr. A.J.R. Habenicht
3. Prof. Dr. W. Sebald

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Zusammenfassung

Die 12/15-Lipoxygenasen (12/15-LOXs) gehören zu einer heterogenen Klasse Lipid-peroxidierender Enzyme, deren biologische Rolle noch nicht vollständig geklärt ist. Eine Reihe experimenteller Daten deuten darauf hin, dass diese Enzym an Reifungs- und Differenzierungsprozessen beteiligt sind und auch für die Pathogenese verschiedener Erkrankungen (Asthma bronchiale, Entzündung, Atherosklerose) bedeutsam zu sein scheinen. Die Expression von 12/15-LOXs wird in vielen Säugetierzellen durch TH_2-Zytokine reguliert und die Zytokin-induzierte Signaltransduktionskaskade verläuft über die Aktivierung van JAK-Kinasen und STAT6. Nach einer Stimulation von A549 Lungenkarzinomzellen mit Interleukin-4 (IL-4) kommt es erst nach 12 Stunden zu einer Hochregulation der 12/15-LOX mRNA Expression. Untersuchungen zum Induktionsmechanismus haben gezeigt, dass Genistein, ein Hemmstoff von Tyrosinkinasen, die Phosphorylierung von STAT6 und dessen Bindung an den Promoter des 12/15-LOX Gens verhinderte. Damit konnte die Induktion der katalytisch aktiven LOX geblockt werden. In Gegensatz dazu verhinderte Zykloheximid, ein spezifischer Hemmstoff der Proteinbiosynthese, die Expression der 12/15-LOX mRNA nicht, Dieses Ergebnis deutet darauf hin, dass die Neusynthese eines Transkriptionsfaktors im Rahmen der IL-4 induzierten Transduktionskaskade unwahrscheinlich ist. Weiterhin wurde beobachtet, dass IL-4 die zelluläre Histonacetyltransferase-Aktivität stark erhöhte und dass dieser Effekt überwiegend auf die enzymatische Aktivität des (CREB-bindenden Protein)-bindenden Proteins (CBP) zurückzuführen ist. Transfektion der Zellen mit E1A, einem viralen Protein, welches als Hemmstoff der Histonacetyltransferase-Aktivität von CBP/p300 bekannt ist, führte zu einer Unterdrückung der 12/15-LOX Expression. Andererseits stimuliert Natriumbutyrat, ein Hemmstoff der Histondeacetylase, die 12/15-LOX Synthese. Damit konnte gezeigt werden, dass die Acetylierung von Histonproteinen und von STAT6 ein essentieller Prozesse bei der IL-4 induzierten 12/15-LOX Expression in A549 Zellen ist. Weiterhin belegen diese Daten, dass sowohl die Phosphorylierung als auch die Acetylierung von STAT6 an der transkriptionellen Aktivierung des 12/15-LOX Gens beteiligt sind, obwohl beide Prozesse eine unterschiedliche Kinetik aufweisen. STAT6 Phosphorylierung erfolgt innerhalb der ersten Stunde nach IL-4 Stimulation, während die Acetylierungsreaktion zeitlich verzögert abläuft. Zusammenfassend kann die

Signaltransduktionskaskade, die in A549 Zellen zur Expression der 12/15-LOX führt, wie folgt beschrieben werden: IL-4 induziert über die Aktivierung von JAK-Kinasen eine Phosphorylierung von STAT6, dessen Bindung an den 12/15-LOX Promotor jedoch zunächst durch nicht-acetylierte Histonproteine verhindert wird. Nach 9-11 Stunden werden Histone und der phosphorylierte STAT6 durch die Acetyltransferase-Aktivität von CBP/p300 acetyliert. Diese Reaktion führt zu einer Veränderung der Histonstruktur, wodurch die Bindung von modifizierten STAT6 und damit die Expression des 12/15-LOX Gens ermöglicht wird.

Als wesentliche zellphysiologische Konsequenz der IL-4 induzierten 12/15-LOX Expression in A549 Zellen, wurde eine Apoptoseinduktion beobachtet. Das endogene 12/15-LOX Produkt 15-HETE bindet an den Kernrezeptor PPARg und induziert damit den programmierten Zelltod. Vorinkubation von A549 Zellen mit dem LOX-Hemmstoff NDGA oder der Einsatz von PPARg Dominant Negativ Vektor verhinderten die Apoptose. Mechanistische Untersuchungen zum Ablauf des durch IL-4 induzierten Zelltodes zeigten, dass der Prozess überwiegend dem extrinsischen Mechanismus folgt, bei dem Kaspasen-8 direkt zu einer Aktivierung der Effektorkaspase-3 führt. Der mitochondriale Mechanismus (Spaltung von Bid bzw. initiale Cytochrom C Freisetzung) scheint dabei nicht involviert zu sein. Die IL-4 induzierte Apoptose könnte von pathophysiologischer Bedeutung für den Verlauf von Lungenerkrankungen sein, bei denen Zellen mit hoher konstitutiver 12/15-LOX Expression, z.B. eosinophile Granulozyten, beteiligt sind.

Hepoxiline sind bioaktive Mediatoren des 12/15-LOX Weges der Arachidonsäurekaskade, die durch Isomerisierung des primären Oxygenierungsproduktes 12S-HpETE gebildet werden. Zu Beginn unserer Untersuchungen war überwiegend unklar, welche Enzyme an der Isomerisierungsreaktion beteiligt sind. Bei der Suche nach geeigneten zellulären Modellen für die Untersuchung dieser Fragestellung fanden wir heraus, dass in den Ratteninsulinom-Zellen Rinm5F, die wegen ihres Mangels an Glutathionperoxidasen eine geringe Kapazität zur Reduktion von 12S-HpETE aufweisen, die Synthese von Hepoxilin A_3 (HXA_3) besonders hoch ist. Da wir vermuteten, dass 12/15-LOXs für die Isomerisierung von 12S-HpETE zu HXA_3 verantwortlich sein könnten, verfolgten wir eine duale Forschungsstrategie um experimentelle Hinweise für

unsere Arbeitshypothese zu finden. In den 12/15-LOX exprimierenden Rinm5F Zellen führte eine Immunopräzipitation mit 12/15-LOX spezifischen Antikörper zu einen vollständigen Verlust der 12/15-LOX- und der Hepoxilinsynthase-Aktivität eines Zelllysates. Beide Aktivitäten wurden fast vollständig im Immunopräzipitat wiedergefunden. 2. Transfektion von HeLa Zellen, die selbst keine 12/15-LOX exprimieren, mit 12/15-LOX und gleichzeitige Hemmung der zellulären Glutathionperoxidasen (Depletion von GSH mit Diethlmaleat) führte zu einer deutlichen zellulären Hepoxilinsynthese. Bei entsprechenden Kontrolltransfektanten wurde diese Aktivität nicht beobachtet. Weiterhin konnte festgestellt werden, dass rekombinante 12/15-LOXs (Expression in E. coli) in vitro eine intrinsische Hepoxinsynthase-Aktivität aufweisen, wenn 12S-HpETE als Substrat angeboten wird. Diese Daten belegen, dass 12/15-LOXs neben den bisher beschriebenen katalytischen Aktivitäten (Oxygenase, Hydroperoxidase, Leukotrienesynthase) auch Hepoxilinsynthase-Aktivität aufweisen.

ABSTRACT

12/15-Lipoxygenases (human 15-LOX-1, rat 12/15-lipoxygenase) belong to family of lipid peroxidising enzymes. The enzyme has been implicated with roles in a variety of pathological conditions such as asthma, atherosclerosis, inflammation and in cellular differentiation. The enzyme expression in most human cell types is tightly regulated by Th2 cytokines, interleukin-4 (IL-4) and interleukin-13 (IL-13). Interleukin-4 (IL-4) induces expression of reticulocyte-type 15-lipoxygenase-1 (15-LOX-1) in various mammalian cells via the Janus kinase/signal transducer and activator of transcription 6 (STAT6) signaling system. 15-LOX-1 mRNA expression was first observed only 12h post IL-4 stimuation and required a minimum of 11h exposure to the cytokine. The mechanism of 15-LOX-1 induction in A549 lung epithelial cells and the observed delay was studied and it was found that genistein, a potent tyrosine kinase inhibitor, prevented phopsphorylation of STAT6, its binding to the 15-LOX-1 promoter, and the expression of catalytically active enzyme. In contrast, cycloheximide did not prevent 15-LOX-1 induction. Surprisingly, it was observed that IL-4 up-regulated the histone acetyltransferase activity of CREB-binding protein (CBP)/p300, which is responsible for acetylation of nuclear histones and STAT6. The acetylation of both proteins appears to be essential for the IL-4-induced signal transduction cascade, because inhibition of CBP/p300 by the viral wild-type E1A oncoprotein abrogated acetylation of both histones and STAT6 and strongly suppressed transcriptional activation of the 15-LOX-1 gene. Moreover, the inhibition by sodium butyrate of histone deacetylases, which apparently suppress 15-LOX-1 gene transcription, synergistically enhanced the IL-4-stimulated 15-LOX-1 expression. These data suggest that both phosphorylation and acetylation of STAT6 as well as acetylation of nuclear histones are involved in transcriptional activation of the 15-LOX-1 gene, although these reactions follow differential kinetics. STAT6 phosphorylation proceeds within the first hour of IL-4 stimulation. In contrast, CBP/p300-mediated acetylation requires 9-11 h, and similar kinetics were observed for the expression of the active enzyme. Thus, the results suggest that in the absence of IL-4, nuclear histones may be bound to regulatory elements of the 15-LOX-1 gene, preventing its transcription. IL-4 stimulation causes rapid phosphorylation of STAT6, but its binding to the promoter appears to be prevented by nonacetylated histones. After 9-11 h, when

histones become acetylated, STAT6 binding sites may be demasked so that the phosphorylated and acetylated transcription factor can bind to activate gene transcription.

The proinflammatory cytokine IL-4 is secreted in large amounts during allergic inflammatory response in asthma and plays a pivotal role in the airway inflammation. IL-4 has been shown to up-regulate 15-lipoxygenase and produce 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) in A549 cells via the Janus kinase/STAT6 pathway under coactivation of CREB binding protein/p300. IL-4 has also been shown to up-regulate peroxisome proliferator-activated receptor (PPARg) nuclear receptors in macrophages and A549 cells. In this study it is observed that 15(S)-HETE binds to PPARg nuclear receptors and induces apoptosis in A549 cells. Moreover, pre-treatment of cells with nordihydroguaiaretic acid, a 15-lipoxygenase inhibitor, prevented PPARg activation and apoptosis. The latter was accomplished by the interaction of the 15(S)-HETE/PPARg complex with the adapter protein Fas-associating protein with death domain and caspase-8, as shown by transfection of Fas-associating protein with death domain dominant negative vector and cleavage of caspase 8 to active subunits p41/42 and p18. Whereas IL-4 and PPARg ligands failed to induce cleavage of Bid and release of cytochrome c from mitochondria, they caused translocation of the proapoptotic protein Bax from cytoplasm to mitochondria with a concomitant decrease in the Bcl-X_L level. The cells were, thereofre, observed to follow the extrinsic pathway of apoptosis where caspase-8 directly activates the effector caspase-3, bypassing the mitochondria. The data also suggests that in IL-4-stimulated cells the 15(S)-HETE/PPARg complex down-regulates Bcl-X_L, and the translocation of Bax to the mitochondria commits the cell to apoptosis. The IL-4-induced apoptosis may contribute to severe loss of alveolar structures and infiltration of eosinophils, mononuclear phagocytes, etc., into the lung tissue as observed in chronic asthma patients.

The 12(S)-lipoxygenase (12-LOX) pathway of arachidonic acid (AA) metabolism after dioxygenation to 12(S)-hydroperoxy-eicosatetraenoic acid is bifurcated in a reduction route to formation of 12(S)-hydroxy-eicosatetraenoic acid (12-HpETE) and an isomerization route to formation of hepoxilins. Interestingly, rat insulinoma RINm5F cells, which are devoid of cytoplasmic glutathione peroxidase (cGPx)/phospholipid hydroperoxide glutathione peroxidase (PHGPx), were observed to produce solely

hepoxilin A_3 (HXA_3). Since HXA_3 synthesis was abolished in heat-denatured or cGPx- or PHGPx-transfected cells, suggesting that a HXA_3 synthase activity regulated by cGPx/PHGPx is present. To confirm this assumption AA was incubated with HeLa cells overexpressing the rat 12/15-LOX. Neither HXA_3 nor 12(S)-HETE were detected due to abundance of cGPx/PHGPx. But, pretreatment of transfected cells with diethyl maleate, an inhibitor of glutathione and PHGPx, restored HXA_3 synthase and 12-LOX activities. Moreover, recombinant rat 12/15-LOX produced HXA3 when incubated with 12-HpETE. Further confirmation was obtained by immunoprecipitation with 12/15-LOX specific antibodies. Immunoprecipitation of Rinm5F lysates results in the depletion of hepoxilin synthase activity. The hepoxilin synthase activity was localised in the immunoprecipitated protein. Thus, cells containing rat 12/15-LOX also possess an intrinsic HXA_3 synthase activity, which is activated by inhibition of cGPx/PHGPx. In normal cells HXA_3 is down-regulated by cGPx/PHGPx, but, it is persistently activated in oxidatively stressed cells deficient in cGPx/PHGPx, such as Rinm5F. Furthermore, formation of corresponding epoxyhydroxy products was observed when 15-HpETE was used as substrate, indicating a broad range of specificity for the enzyme.

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ABBREVIATIONS

AA	Arachidonic Acid
ADAM reagent	9-anthryl diazomethane
AIF	Apoptosis inducing factor
AP-2	Activator Protein – 2
APAF-1	Apoptotic protease activating factor-1
Bad	Bcl-2/Bcl-XL associated death promoter
Bax	Bcl-2 associated protein
Bcl-2	B cell leukemia-2
Bcl-X_L	B cell leukemia X-long
Caspase	Cysteine aspartyl-specific protease
CBP	cAMP response element binding protein binding protein
CD-95, CD36	Cluster of Designation-95, 36
cGPx	Cytoplasmic glutathione peroxidase
CHX	cycloheximide
COPD	Chronic obstructive pulmonary disease
DAXX	Death associated protein
DIABLO	Direct IAP binding protein
DICE	Differentiation control elements
DiHpETE	Dihydroperoxy eicosatetraenoic acid
DISC	Death-inducing signalling complex
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
et al	et alii
FADD	Fas-associating protein with death domain
FasL	Fas ligand
GC-MS	Gas chromatography-mass spectrometry
GI-GPx	Gastrointestinal-glutathione peroxidase
GPx	Glutathione peroxidase
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised form)
GST	Glutathione transferase
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HEPES	(N-[2-hydroxyethyl]piperazine-N‘-[4-butanesulfonic acid])
HETE	Hydroxy eicosatetraenoic acid
HMG-1	High mobility group-1
hnRNP	Heterogeneous nuclear ribonucleoproteins
HODE	Hydroxy octadecadienoic acid
HpETE	Hydroperoxy eicosatetraenoic acid
HPLC	High performance liquid chromatography
HpODE	Hydroperoxy octadecadienoic acid
HxA_3, B_3	Hepoxilin A_3, B_3
IAP	Inhibitor of apoptosis protein
IFN□	Interferon □
IL-13	Interleukin – 13
IL-1□	Interleukin-1□
IL-4	Interleukin – 4
JAK 1/2	Janus tyrosine kinase-1/2

LA	Linoleic Acid
LDL	Low density lipoprotein
LOX	Lipoxygenase
LTA4	Leukotriene A4
NDGA	Nordihydroguaiaretic acid
NF-□B	Nuclear factor kappa b
NO	Nitric oxide
ONPG	ortho-nitrophenyl-para- D galactopyranoside
OPP	4-(2-oxapentadeca-4-yne)phenylpropanoic acid
oxLDL	Oxidised low density lipoprotein
p90RSK	p90 Ribosomal S6 kinases
PBS	Phosphate buffered saline
PCAF	CBP/p300-associated factor
PHGPx	Phospholipid hydroperoxide glutathione peroxidase
PMSF	Phenylmethylsulphonyl fluoride
PPAR	Peroxisome proliferator-activated receptor
PUMA	p53 upregulated modulator of apoptosis
Rb	Retinobalstoma
RIP	Receptor interacting protein
RT-PCR	Reverse transcriptase polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SIMPs	Soluble intermembrane proteins
SMAC	Second mitochondria-derived activator of caspase
SOCS	Suppresser of cytokine signalling
SRC-1	Steroid receptor coactivator-1
STAT6	Signal transducer and activators of transcription-6
TAF(II)250	TATA-box binding protein (TBP) associated factor (II) 250
TCPO	Trichloropropene oxide
TdT	Terminal deoxynucleotide transferase
TEMED	N,N,N‘,N‘-tetramethylethlenediamine
TLC	Thin layer chromatography
TNFR-1	TNF receptor-1
TNF□	Tumor Necrosis Factor □
TRADD	TNFR-1-associated death domain protein
TRAIL	TNF related apoptosis inducing ligand
Tris	Tris(hydroxymethyl)aminomethane
$TrXA_3$, B_3	Trioxilin A_3, B_3
TUNEL	Terminal deoxynucleotide transferase nick end labelling
Tyk-2	Tyrosine kinase-2 (Janus family)
VDAC	Voltage dependent anion channel

1. INTRODUCTION

1.1 Lipoxygenases as lipid peroxiding enzymes.

Lipoxygenases form a family of non-heme iron containing enzymes, which dioxygenate polyunsaturated fatty acids at specific postions to produce a series of hydroperoxy fatty acids. A variety of substrates such as free fatty acids, membrane phospholipids and lipoproteins can be used as substrates for peroxidation by the lipoxygenases. These enzymes specifically recognise the 1-cis, 4-cis pentadiene structures to introduce molecular dioxygen and create a cis-trans conjugated diene. The most accepted hypothesis for the lipoxygenase reaction is the radical hypothesis (Kühn *et al.*, 1986b; Kühn and Thiele, 1999) according to which the reaction consists of three major steps (figure 1):

a) hydrogen is abstracted from the bisallylic methylene. The specificity of the hydrogen abstraction, both positional- and stereo-selectivity (-S, -R), depends upon the orientation of the substrate at the active site and by the specificity properties of the enzyme.

b) re-arrangement of the radicals leading to the formation of a cis-trans conjugated diene system. The direction of the rearrangement ([-2] or [+2]) is determined again by the orientation in the active site.

c) the molecular dioxygen is inserted sterospecifically forming a peroxy radical, which is subsequently reduced to hydroperoxide anion.

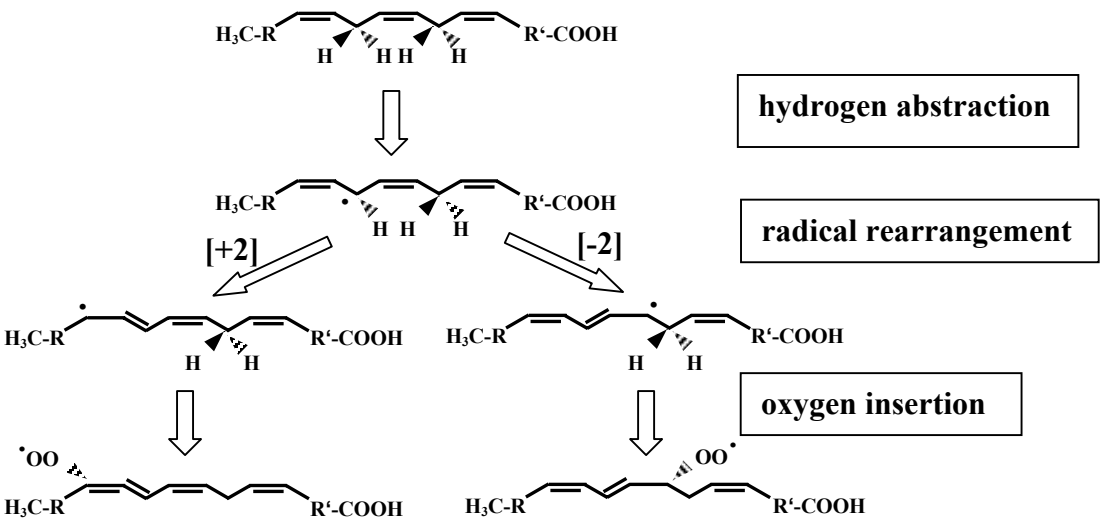


Figure 1. Radical mechanism of the lipoxygenase reaction

1.2 Diversity of the lipoxygenase family

This family of enzymes is widely distributed in the animal and plant kingdom (Kühn and Thiele, 1999; Brash, 1999, Mack *et al.*, 1987; Grechkin, 1998). Lipoxygenases have also been discovered in lower organisms, such as corals (Brash *et al.*, 1996), fungi (Bisakowski *et al.*, 1997; Su and Oliw, 1998) and even bacteria (Porta and Rocha-Sosa, 2001). The nomenclature of the enzyme is based upon the number of the carbon atom which is preferentially oxygenated in arachidonic acid (AA). The positional nomenclature is further specified by mentioning the tissue source of the lipoxygenase e.g. leukocyte, platelet, and the stereospecificty by adding S and R to the name. This system is, however, fraught with difficulties. The 12-lipoxygenases of leukocyte type share only 40% homology with the 12-lipoxygenase of platelet type. On the other hand they are 75% similar to 15-lipoxygenase of the reticulocyte type (Yamamoto, 1992). Phylogenetic classification (Kühn and Thiele, 1999) of the various mammalian lipoxygenases leads to the subdivision of these enzymes into 4 main subtypes (figure 2): 12/15 lipoxygenases, 5 lipoxygenases, 12 lipoxygenase of the platelet type and the epidermis type lipoxygenases. This has been proven to be a more rational system since enzymes with similar sequence, structure and activity are grouped together like 15-LOX and 12-LOX leukocyte type.

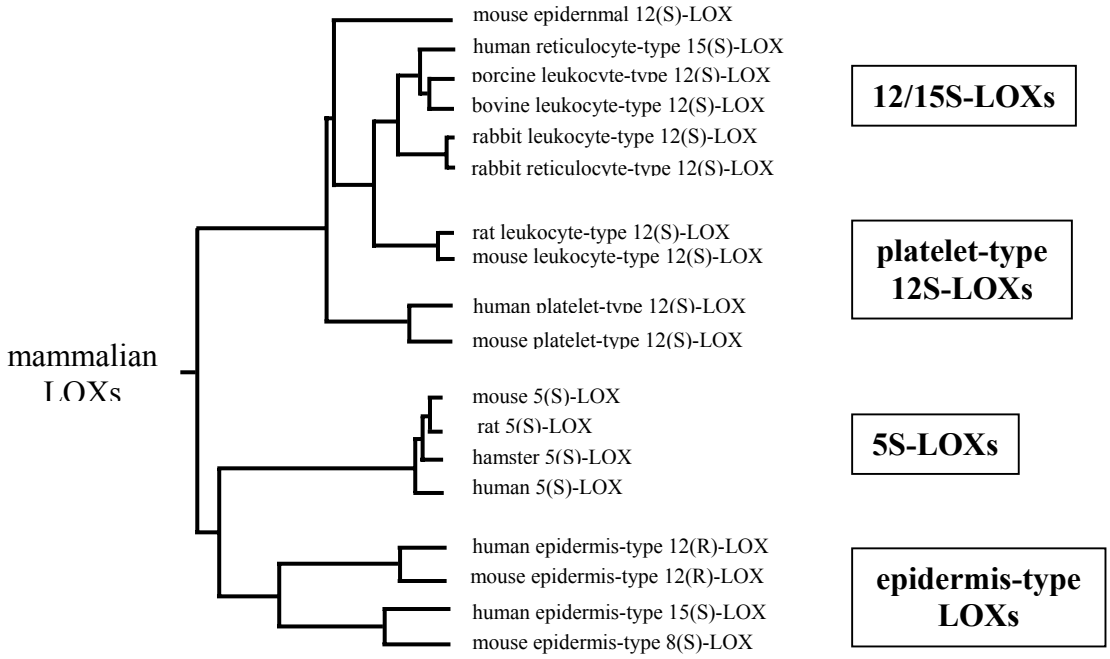


Figure 2. Phylogenetic tree of mammalian lipoxygenases.

1.3 12/15 lipoxygenases: Protein chemical and enzymatic properties

So far the 12/15-lipoxygenase (12/15-LOX) family consists of murine, rat, bovine and porcine leukocyte 12-lipoxygenases, rabbit reticulocyte 15-lipoxygenase (15-LOX) and human 15-lipoxygenase-1 (15-LOX-1). These enzymes share a remarkable degree of homology with each other both at the protein, and DNA level. They also exhibit many common enzymatic properties such as broad range of substrate specificity and reaction kinetics (Yamamoto, 1992). To date, the simultaneous expression of these enzymes in the same species has been observed only in rabbits (Berger *et al.*, 1998) leading to the hypothesis that leukocyte type 12-LOX in other species may be functionally complementary to the 15-LOXs in rabbits and humans.

A 15-LOX was first observed in rabbit reticulocytes where it was shown to play an important role in the maturation of reticulocytes to erythrocytes (Schewe *et al.*, 1975; Rapoport *et al.*, 1979). Later, this enzyme was also described in human eosinophils (Sigal *et al.*, 1988) and bronchial epithelial cells (Henke *et al.*, 1988; Nadel *et al.*, 1991). The enzyme consists of 663 amino acids, 75 kDa, and the degree of similarity between the two species is more than 99%. In pigs, it was first purified from leukocytes (Yoshimoto *et al.*, 1982), in mice from spleen (Chen *et al.*, 1994) and in rat brain (Watanabe *et al.*, 1993). The murine and porcine enzymes share a high degree (about 75%) of sequence homology with rabbit and human 15-LOXs. 12/15-LOX enzymes exhibit a broad substrate specificity. In contrast to other lipoxygenases, they are capable of oxygenating free polyunsaturated fatty acids like arachidonic acid (AA), linoleic acid (LA) and linolenic acids, esterified lipids like mono, di and tri-acylglycerols, cholesterol esters and phospholipids as well as complex substrates like biomembranes and lipoproteins (Rapoport *et al.*, 1979; Schewe *et al.*, 1986). These enzymes exhibit dual positional specificity with arachidonic acid. The ratio of 12- to 15-HETE varies between the lipoxygenases from different species (Yoshimoto and Yamamoto, 1995). The reaction proceeds by the abstraction of a hydrogen from the bisallylic methylene at the C-10 position (12-LOX) or at the C-13 (15-LOX) followed by insertion of oxygen. Both the processes occur antarafacially i.e. from different sides of the plane of the double bonds.

1.3.1 Tissue distribution

The tissue distribution of 12/15-LOXs varies in different species. In rat, the 12-leukocyte type enzyme is maximally expressed in the pineal gland followed by leukocytes, macrophages, lung, spleen, pituitary, pancreatic beta cells and aorta, while in mouse, the maximum

expression was seen in the peritoneal macrophages, kidney and pineal gland with weaker signal observed in intestine, spleen and aorta (Yoshimoto and Takahashi, 2002). Simultaneous expression of both 12(S) leukocyte type and 15-LOX was observed in rabbits (Berger *et al.*, 1998). The 12-leukocyte type enzyme was detected in the tracheal epithelium and leukocytes of cows and in the pitutary gland and leukocytes of pigs. 15-LOX-1 is the only 12/15-LOX isoform which has been detected in humans. It is expressed constitutively in bronchial epithelium and eosinophils (Sigal *et al.*, 1988) though expression is also observed in monocytes and colonic epithelium upon cytokine (IL-4) stimulation.

1.3.2 Suicide inactivation

Another interesting feature of this enzyme is the property of suicide inactivation. Reaction with fatty acids rapidly slows down and stops within several minutes of incubation (Rapoport *et al.*, 1984; Hada *et al.*, 1991). This is due to a complex set of mechanisms not yet clearly understood. Kishimoto *et al.*, 1996, have shown that 15(S)-HpETE produced as product of AA metabolism covalently binds to the 12-LOX leukocyte protein and inactivates the enzyme. This feature has also been observed from the rabbit 15-LOX. Epoxide products of 15-HpETE, such as 14,15-LTA_4 involve the formation of radical intermediates, which were hypothesised to covalently link to active site residues causing enzyme inactivation. Inhibition was also observed with LA independent of covalent linkages suggesting the proposed LTA_4 mechanism may not be exclusive (Wiesner *et al.*, 2003).

1.3.3 Structural requirements for 12/15 lipoxygenation

Among the mammalian lipoxygenases, the X-ray crystal structure is available only for the rabbit 15-LOX (Sloane *et al.*, 1990; Gillmor *et al.*, 1997). Much of the information about structure and function of this protein has been elucidated utilising the crystal structure data along with site-directed mutagenesis and modified substrates. The enzyme is composed of a large C- terminal catalytic domain containing the non-heme iron and a small N-terminal beta barrel domain. The substrate binding site is a large boot shaped hydrophobic cavity, which contains the non-heme iron catalytic centre (Gillmor *et al.*, 1997). The active site is lined mainly by the hydrophobic side chains of amino acids. Phe 353, Ile 418, Met 419, Ile 593 form the bottom of substrate binding cavity and a positively charged Arg 403 is positioned at the entrance of the active site. It has been proposed that the substrate fatty acid slide into the active site with its methyl end ahead and the positively charged Arg 403 interacts with the carboxy group forming a salt bridge (Gan *et al.*, 1996). Replacement of this residue with a

neutral amino acid residue severely impaired the dioxygenation process. Inside the active site the methyl end interacts with various side chains to position the bisallylic methylene C-13 of the substrate close to the iron atom for hydrogen abstraction. Site-directed mutagenesis studies have revealed that Phe 353 (Borngräber *et al.*, 1996), Ile 418, Met 419 and Ile 593 (Sloane *et al.*, 1991; Borngräber *et al.*, 1999) are the positional determinants of 15 lipoxygenation (C-13 abstraction) single or combined. Mutation to these amino acids to residues with less bulkier side chains (alanine) converted the enzyme to a 12-LOX. The overall cavity size of 12-LOX leukocyte type was predicted to be moderately bigger, allowing the substrate to slide in deeper and aligning the C-10 of AA atom for hydrogen abstraction at the non-heme iron.

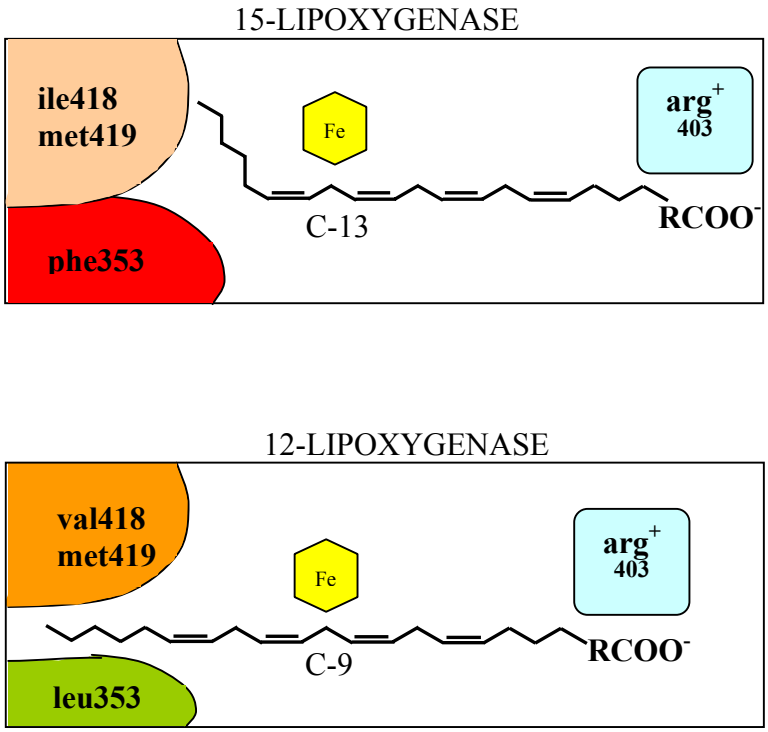


Figure 3. Position determinants of lipoxygenase reaction.

This suggests that the space inside the active site cavity plays an important role in the positional specificity (Borngräber *et al.*, 1999). The reverse process on 12-LOX works equally well (Suzuki *et al.*, 1994; Watanabe and Haeggstrom, 1993). However, conversion to 5-LOX by mutagenesis has not been successful. The positional determinant residues on 15-LOX were mutated to those of 5-LOX but the enzyme was inactive (Sloane *et al.*, 1990). 15-LOX possess the ability to oxygenate 15-HpETE to form 5, 15-diHpETE. Methylation of carboxy end of the substrate increased the activity significantly. This phenomenon was hypothesised to be due to an inverse orientation of the substrate at the active site. In this case the caroboxy end may slide into the cavity as suggested by experiments with modified

substrates and site directed mutagenesis (Schwarz *et al.*, 1998; Walther *et al.*, 2001). Thus, the determinant of positional specificity is not only the volume but also the orientation of the substrate in the active site.

The N-terminal domain of the enzyme does not play a major role in the dioxygenation reaction of 12/15 lipoxygenase. N-terminal domain truncations did not impair the lipoxygenase activity. The ability of the enzyme to bind to membranes, however, is impaired in the mutants (point and truncations) of the N-ternimal domain without significant alterations to the catalytic activity (Walther *et al.*, 2002). Mutation to Trp 181, which is localised in the catalytic domain, also impaired membrane binding function. This suggests that the C-terminal domain is responsible for the catalytic activity and a concerted action of N-terminal and C-terminal domain was necessary for effective membrane binding.

1.4 Regulation of 12/15-LOX expression

The expression of 15-LOX can be controlled at the transcriptional, translational, post-translational levels (Kühn *et al.*, 1999, Kühn *et al.*, 2002).

1.4.1 Transcriptional regulation

1.4.1.1 Rabbit 15-LOX: Constitutive expression of rabbit 15-LOX is observed only in reticulocytes under the condition of experimental anemia, where it is strongly expressed in peripheral monocytes, lung, liver, spleen and kidneys (Schewe *et al.*, 1975; Rapoport *et al.*, 1979). The mechanism of induction of 15-LOX during experimental anemia is still unclear. O'Prey and Harrison, 1995, studied the promoter region of the gene and found a number of negative and positive regulatory elements, which are differentially regulated in erythroid (15-LOX expressing) and non-erythroid cell lineages (non-expressing). In 1 Kb of 5' region flanking the 15-LOX gene, they observed multiple copies of a putative transcriptional silencer element, which function only in non-erythroid cell lineages. DNAse I mapping studies revealed protein binding to these elements only in non-erythroid cells and not in erythroid cells. This element was therefore suggested to function like a silencer element in non-erythroid cells, inhibiting the transcription of the gene. Several other positive regulatory elements were observed in erythroid cells, which could putatively bind to transcription factors such as GATA proteins.

1.4.1.2 Human 15-LOX-1: In humans, the Th2 cytokines, IL-4 and IL-13 have been shown to upregulate the expression of this gene in peripheral monocytes (Conrad *et al.*, 1992), A549 lung epithelial carcinoma cells (Brinckmann *et al.*, 1996), human tracheobroncheal cells (Jayawickreme *et al.*, 1999), endothelial cells (Lee *et al.*, 2001) and in Caco-2 colon

carcinoma cells (Kamitani *et al.*, 2000). Human alveolar macrophages appear to constitutively express 15-LOX-1 in low levels as indicated by activity assays (Levy *et al.*, 1993). Other cytokines did not induce the enzyme. In fact, the Th1 cytokine IFNγ was observed to inhibit gene expression induced by IL-13 in monocytes (Nassar *et al.*, 1994). The IL-4 and IL-13 pathway share JAK1/2 kinases and transcription factor STAT6 (Heim, 1999). IL-4 and IL-13 failed to elicit any response in monocytes prepared from STAT6 deficient mice (Heydeck *et al.*, 1998) and in human monocytes antisense treatment showed the involvement of JAK2 and TYK2 kinases in the upregulation (Roy and Cathcart, 1998). Pretreatment of the cells with IFNγ inhibited the phosphorylation of these kinases and stopped the signal transduction cascade. Involvement of factors such as SOCS (suppressors of cytokine signalling) have implicated in this process (Dickensheets *et al.*, 1999).

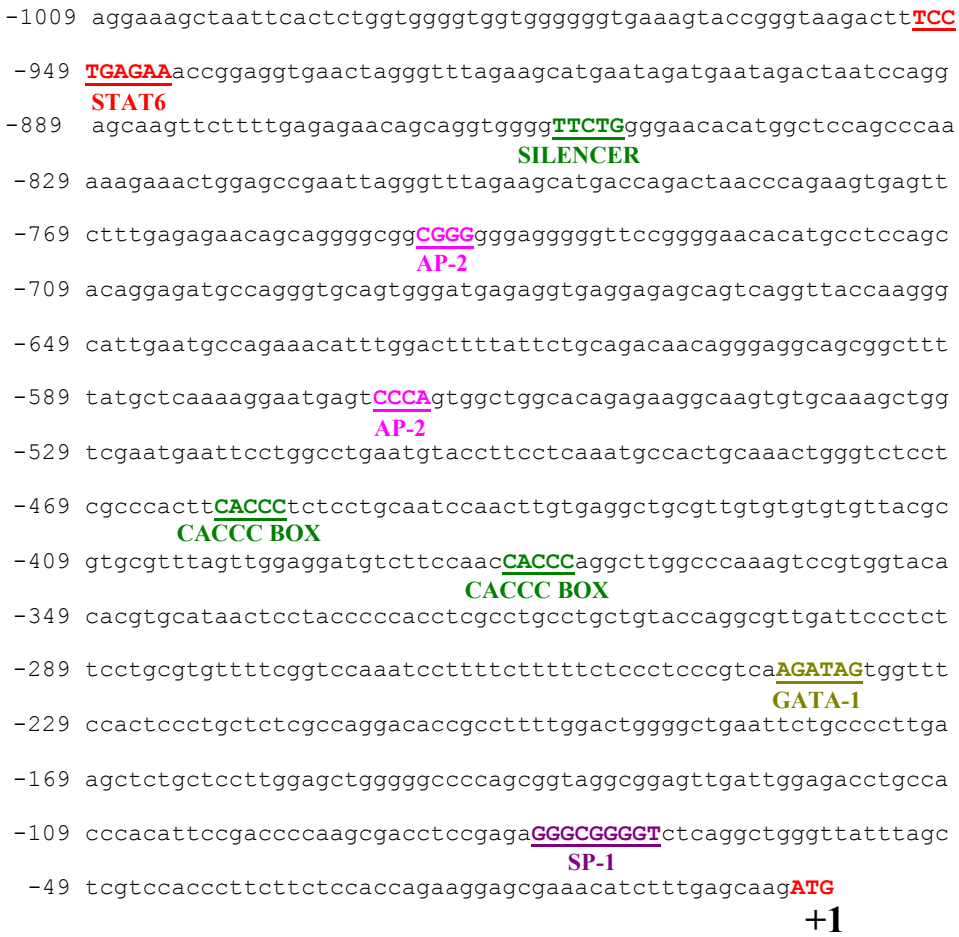


Figure 4. Promoter region of human 15-LOX-1 showing putative binding sites for transcription factors

The promoter of 15-LOX-1 revealed a putative STAT6 binding site and mutation of this site (-952) abolished the IL-4 induced 15-LOX-1 expression (Conrad and Lu, 2000; Kritzik *et al.*,

1997). Several other prominent transcription factor binding sites have been identified in this promoter region. A single copy of the "silencer element" identified in rabbit 15-LOX is present in the human promoter, however its function has not been elucidated. Kevalkar *et al.*, 1998, have identified a 29 bp region (-352 to -304) which is important for the cytokine induced expression. Deletion of this region resulted in the absence of expression. Ku 70/80 has been identified as the protein binding to this region and controlling the gene expression (Kelavkar *et al.*, 2000b). In Caco-2 cells, histone acetylation has been shown to play an important role. Cells treated with sodium butyrate, a hisone deacetylase inhibitor, expressed 15-LOX-1 without cytokine stimulation (Kamitani *et al.*, 2001). Histone remodelling is a well known regulator of gene transcription and adds an interesting dimension to the study of transcription regulation of 15-LOX-1.

1.4.1.3 Leucocyte type 12-LOX: Expression of 12/15-LOX was induced in porcine smooth muscle cells (Natarajan *et al.*, 1996) and murine system by IL-4 and IL-13 (Conrad *et al.*, 1992), however, the involvement of STAT6 is not clear. No induction of 12/15-LOX expression by IL-4 was observed in peritoneal macrophages from STAT6 deficient mice (Heydeck *et al.*, 1998) but the constitutive expression of the enzyme was not affected (Sendorby *et al.*, 1998). Another cytokine IL-1β has been observed to upregulate rat 12/15-LOX expression in rat insulinoma cells both at the mRNA and protein levels (Bleich *et al.*, 1995). Other authors have suggested that IL-1β induced expression of 12/15-LOX depends on an increased availability of substrate and on the cellular nitric oxide levels (Ma *et al.*, 1996). Melatonin, a hormone involved in seasonal control of metabolism, significantly downregulated 12/15-LOX activity in pineal gland. It has been suggested that melatonin acts as an endogenous modulator of the 12 Lipoxygenase protein (Zhang *et al.*, 1999). Though, a number of molecules appear to modulate the activity and expression of 12/15-LOX, the mechanisms or the signal transduction pathways responsible have not been elucidated in detail.

1.4.2 Translational regulation:

Translational regulation of 12/15 lipoxygenase expression has been investigated in detail in rabbit reticulocytes (Thiele *et al.*, 1982), where it plays a major role in the maturation of the cell to erythrocyte. Abundant quantities of 15-LOX mRNA is present in immature rabbit reticulocyte but no active LOX enzyme could be detected. During maturation, the protein is expressed which by acting specifically on the mitochondrial membranes and promotes organelle degradation (Schewe *et al.*, 1975). This process is controlled by translational

silencing by hnRNP k and E1 (Ostareck *et al.*, 1997). These two RNA binding proteins bind to specific elements known as DICE (differentiation control elements) present in the 3'untranslated region of the 15-LOX mRNA . These elements consists of poly UAA stretches repeated several times. In cell free system, the hnRNP proteins specifically inhibit the assembly of 80S ribosome on the 15-LOX mRNA, thus preventing translation (Ostareck *et al.*, 2001). As the process of maturation proceeds, the two proteins detach from the mRNA, thus permitting the translation to proceed.

1.4.3 Post-translational regulation:

1.4.3.1 Hydroperoxide tone: A major factor in the regulation of the 12/15 LOX activity is the hydroperoxide tone, which quantifies the sum of all hydroperxides present in the cell (Weitzel and Wendel, 1993). Hydroperoxides activate LOXs by converting the non-heme iron from the ferrous to a ferric form. Inhibition of this conversion, by specific inhibitor like OPP, prevents the catalytic action of these enzymes (Richards *et al.*, 1999). *In vivo* the hydroperoxide tone in the cell is regulated by glutathione dependent peroxidases. These enzymes convert hydroperoxy fatty acid to the hydroxide form, and this is paralleled by oxidation of GSH to GSSG (Hurst *et al.*, 2001).

1.4.3.1.1 Selenoenzymes: The Glutathione peroxidase (GPx) family consists of 4 major subfamilies: i) classical intracellular glutathione peroxidase (GPx-1), ii) plasma GPx, iii) gastro-intestinal Gpx (GI-GPx) and iv) phospholipid hydroperoxide GPx (PHGPx) (Flóhe *et al.*, 1973; Takahashi *et al.*, 1987; Chu *et al.*, 1993). The common structural feature of these enzymes is presence of a selenocysteine residue. Of these, GPx-1 and PHGPx are the best characterised isoforms and they are found in almost all cell types. GPx-1 is a 26 kDa cytosolic protein, which exists as a homotetramer and utilises intracellular free hydroperoxy fatty acids as substrates (Flóhe, 1989). PHGPx, on the other hand, is a 23 kDa protein, which exists as a monomer and has the ability to react not only with free hydroperoxy fatty acids but also with esterified hydroperoxy fatty acids. It is preferentially associated with the membranes (Ursini *et al.*, 1985; Thomas *et al.*, 1990; Roveri *et al.*, 1994).

These enzymes constitute an important part of the cellular anti-oxidant defence system. Overexpression of PHGPx has been observed to reduce apoptosis caused by oxidative stress in rat basophilic leukemia cells (Imai *et al.*, 1996). The mitochondrial form of the enzyme has been found to be particularly effective as indicated by inhibition of apoptosis and increase in the level of Bcl-2, a mitochondrial anti-apoptotic protein (Nomura *et al.*, 1999). 12/15-LOX can utilise membrane phospholipids as substrates for oxygenation. Pre-incubation of

membranes with PHGPx inhibited the oxygenation reaction, which was reversed by the addition of hydroperoxides (Schnurr *et al.*, 1996). IL-4 and IL-13 treatment of A549 cells causes induction of 15-LOX-1 but also a concomitant decrease in the levels of PHGPx (Schnurr *et al.*, 1999). Analysis of various tissues of mice overexpressing IL-4 revealed a negative correlation between the PHGPx levels and increased arachidonic acid oxygenase activity in certain tissues. This observation suggests that lipid peroxide generating and reducing enzymes are inversely regulated in various mammalian cells. It has been reported, that PHGPx is primarily responsible for the regulation of 5-lipoxygenase activity in rat leukocyte (Weitzel and Wendel, 1993).

A major reduction in the glutathione peroxidase activity was observed in the platelets from selenium deficient rats (Bryant *et al.*, 1980). These platelets produced at least seven fold higher amounts of 12-HpETE as compared to control rats ((Bryant *et al.*, 1983). Similar results were obtained in leukocytes from selenium deficient rats (Ho *et al.*, 1997). Moreover, the selenium deficient rat platelets showed a three fold increase in the synthesis of isomeric trihydroxy fatty acids, $TrXA_3$ and $TrXB_3$ (Bryant *et al.*, 1983). The isomerization pathway produces HXA_3 and HXB_3 from 12 HpETE which are rapidly hydrolysed by epoxide hydrolases to $TrXA_3$ (Pace-Asciak and Lee, 1989). As the reductase activity of glutathione peroxidase is limited in selenium deficient rat platelets, the metabolism of 12-HpETE is diverted towards the isomerization route. This suggests the importance of glutathione peroxidases in the reduction of 12-HpETE. These authors, however, assume that the classical glutathione peroxidase, GPx-1 is the sole selenium dependent glutathione peroxidase enzyme present in platelets. However, platelets from GPx-1 knockout mice incubated with 25 µM arachidonic acid still synthesised 12-HETE (Ho *et al.*, 1997). Furthermore, Gpx-1 deficient mice exhibited no phenotypic changes and the rate of lipid peroxidation and consumption of exogenous H_2O_2 was not altered in the tissues when compared with normal mice. This implies the ability of other enzyme(s) to take over the function of GPx-1, namely PHGPx. Selenium depletion studies also revealed a different response pattern between GPx-1 and PHGPx (Bermano *et al.*, 1995). Severe selenium deficiency in rats caused complete loss of GPx-1 activity and GPx-1 mRNA levels in liver and heart, while the PHGPx activity was reduced by only 75%, and the mRNA levels were unaffected. In selenium-deficient rat basophilic leukemia cells, <1% GPx activity and 35% PHGPx activity were observed as compared to control cells and upon activation an enhanced 12-lipoxygenase activity was observed. Replenishment of selenium to these cells restored the PHGPx activity within 8 h, while the GPx-1 activity needed 7 days to return to normal levels (Weitzel and Wendel,

1993). The role of PHGPx and GPx-1 in the regulation of hydroperoxide tone seems to be different. An important factor for consideration is the localisation of the enzymes. PHGPx is preferentially associated with the membranes, which is also the site of eicosanoid synthesis. Similar observation has been reported in human platelets where PHGPx may have a special function in the regulation of 12-LOX activity (Sutherland *et al.*, 2001).

1.4.3.1.2 Selenium independent systems: Using a sensitive HPLC assay, glutathione transferases (GST) from α, μ and θ groups were observed to reduce phospholipid hydroperoxides to corresponding hydroxides using GSH as the reducing agent (Hurst *et al.*, 1997). The α group exhibited the maximum activity, though it was significantly lesser than that of PHGPx. Also, in contrast to the glutathione peroxidases, these enzymes were inactive in the presence of detergent, Triton X100 (Hurst *et al.*, 1998). The π enzymes were, however, inacapable of reducing phospholipid hydroperoxides. Although, the activity of the GSTs is significantly lower than that of the glutathione peroxidases, their relative abundance (nearly 5% of total cellular protein) could be important. The activity of this class of enzymes with free fatty acid hydroperoxide, however, has not been reported so far.

Recently, a novel non-selenium glutathione peroxidase has been identified. The enzyme, 1-Cys peroxiredoxin, was first isolated from the bovine ciliary body in the olifactory mucosa (Shichi and Demar, 1990) and has been shown to catalyze the reduction of both free fatty acid hydroperoxides and phospholipid hydroperoxides using glutathione as electron donor (Fisher *et al.*, 1999). This enzyme had no glutathione transferase activity when assayed with a spectrum of potential SH donors (Shichi and Demar, 1990). Furthermore, 1-Cys peroxiredoxin also exhibited PLA_2 like activity and both the activities were reported to reside in distinctive active sites (Chen *et al.*, 2000).

1.4.3.2 Other factors: Nitric oxide has been shown to regulate the activity of 12/15-LOXs *in vitro*. Short incubations of the enzyme with NO considerably increases the kinetic lag phase which was shortened upon long term incubations (Holzhütter *et al.*, 1997). Together with EPR and X-ray absorption studies, these data suggest that during NO-LOX interaction oxidation of ferrous non-heme iron may have occurred (Wiesner *et al.*, 1996).

1.4.3.3 Regulation by calcium: 12/15-LOXs exhibit the capability to oxidise biomembranes. *In vitro* reticulocyte-type 15-LOX was observed to bind reversibly to biomembranes such as, submitochondrial particles and erythrocyte ghosts (Brinckmann *et al.*, 1998). This membrane

binding activity was enhanced by the addition of calcium. Interestingly, the addition of calcium also enhanced the membrane oxygenase activity of the enzyme. This feature was confirmed *in vivo*. In rabbit reticulocytes and IL-4 stimulate peripheral monocytes, electron microscopic studies showed the localisation of the enzyme to the plasma and intracellular vesicles. Calcium ionophore augmented the membrane binding share of 15-LOX in human eosinophils, where it is expressed constitutively. Only under these conditions were specific lipoxygenase products of membrane lipids observed membrane oxygenase activity observed (Brinckmann *et al.*, 1998). Thus, calcium not only enhances the membrane translocation but also the catalytic activity of 15-LOX in hematopoetic cells. Similar observations were made in colon carcinoma, Caco-2, cells. The presence of calcium was essential for the translocation and the catalytic activity of the 15-LOX-1, which was overexpressed in these cells (Hsi *et al.*, 2001).

1.5 Metabolites of 12/15-LOX pathway

The predominant product of AA and LA (the major polyunsaturated fatty acids in mammalian cells) metabolism is 12-HpETE, 15-HpETE and 13-HpODE. These metabolites can be further processed into 3 primary types of products.

1.5.1 Peroxide reduction pathway: 12-HpETE, 15-HpETE and 13-HpODE can be converted to the corresponding hydroxides by the action of glutathione peroxidases.

1.5.2 Double and triple oxygenation pathway (Oxygenase activity): Lipoxin B4 (5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid) is a product of the 15 lipoxygenase (Kühn *et al.*, 1987). Lipoxin B4 is a biologically important compound. It is involved in the activation of leukocytes and the inhibition of neutrophil recruitment, chemotaxis and adhesion (Fierro *et al.*, 2002). Lipoxins, especially aspirin triggered 15-epi-lipoxin A4, are potent inhibitors of acute inflammation (Takano *et al.*, 1997).

1.5.3 Epoxy-leukotriene pathway (LTA_4 synthase activity): A concerted action of the oxygenase and hydroperoxidase activty leads to the formation epoxy-leukotrienes such as 14,15 epoxyleukotriene A4 (Bryant *et al.*, 1985).

1.5.4 Lipohydroperoxidase pathway (Hepoxilin synthase activity): The hydroperoxy group is homolytically cleaved to alkoxy radical initiating the formation of epoxyhydroxy compounds (involving the hydroperoxidase activity), keto-dienes, aldehydes and alkanes (Veldink *et al.*, 1997). Of these, the epoxyhdroxy compounds of 12-LOX , hepoxilins, have been best studied (Pace-Asciak and Asotra, 1989).

1.5.4.1 Hepoxilins: Hepoxilins are monohydroxy-epoxy derivatives of arachidonic acid which are products of 12-HpETE. Two forms of hepoxilin are known, HxA_3 (8(R) and 8(S)-hydroxy-11(S),12(S)-epoxyeicosa-5Z,9E,14Z-trienoic acid and HxB_3 (10(R) and 10(S)-hydroxy-11(S),12(S)-epoxyeicosa-5Z,8Z,14Z-trienoic acid) (Pace-Asciak *et al.*, 1983). The intra molecular re-arrangement of 12-HpETE can proceed by two main pathways.

12(S)-HpETE undergoes heme-dependent isomerization to produce HxA_3 and HxB_3 in equimolar amounts (Pace-Asciak, 1984a; Pace-Asciak and Asotra, 1984; Pace-Asciak, 1984b). Incubation of a racemic mixture of 12(R/S)-HpETE with hemin produced an equimolar mixture of HxA_3 and HxB_3 and the R derivatives produced the corresponding hepoxilins which do not occur *in vivo* (Pace-Asciak *et al.*, 1995). This reaction was insensitive to heat. Both the insensitivity to heat and lack of substrate selectivity suggested that hemin catalysed reactions were non-enzymatic. Bryant *et al.*, 1980; 1983, observed that platelets from selenium deficient rats produced more 12-HpETE and tri-hydroxy derivatives upon incubation with AA. This product was identified as a mixture of isomers of Trioxilin A_3 ($TrXA_3$), hydrolysis product of HxA_3.

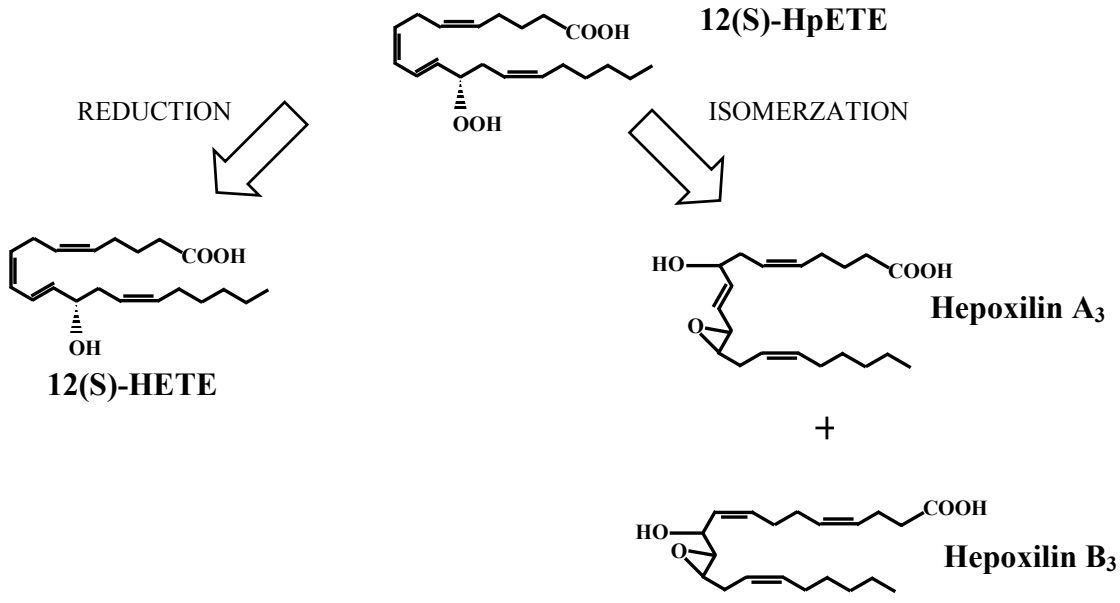


Figure 5. Bifurcation of the 12-Lipoxygenase pathway.

Sutherland *et al.*, 2001, have demonstrated that platelets produce an equimolar mixture of HxA_3 and HxB_3 from 12-HpETE. The inability of the earlier authors to observe both the hepoxilins in platelets has been attributed to the instabilty of hepoxilins in acidic extraction conditions (Pace-Asciak, 1994a). Similar observations were made in trout gills (German and

Kinsella, 1986). Rat pineal gland slices, on the other hand incubated with 12(R/S)-HpETE produced exclusively HxA_3. Here 12(S)-HpETE was selectively consumed (Reynaud *et al.*, 1994). Tissue, boiled prior to use, did not produce any hepoxilins. Taken together, these data indicate the presence of a heat labile enzyme which stereoselectively converts 12(S)-HpETE to HxA_3. Similar reaction was also observed in rat brain and skin (Pace-Asciak *et al.*, 1993). This enzyme, designated as "hepoxilin synthase", did not seem to require any cofactors.

Another probable pathway for the production of hepoxilins is the cytochrome P450 pathway. Incubation of 15(S)-HpETE with rat liver microsomes produced the mono-hydroxyepoxy derivative, 11S-hydroxy-14S,15S-trans-epoxyeicosa-5Z,8Z,12E-trienoic acid (Weiss *et al.*, 1987). 15(R)-HpETE was converted into the corresponding 15R derivative. The P450 2B1 enzyme gave products similar to those obtained from the purified microsomes. Similar derivatives were also observed upon the incubation of 15-HpETE with garlic root tips (Reynaud *et al.*, 1999). Reticulocyte 15LOX, under anaerobic conditions, produces significant amounts of a 13-hydroxy-14,15-epoxy eicosatrienoic acid. This compound was also observed under aerobic conditions but to a significantly lesser extent (Kühn *et al.*, 1986a). In algae, another enzyme has been isolated which produces epoxy hydroxy derivatives from free fatty acids (Moghaddam *et al.*, 1990).

HxA_3 is chemically and biologically unstable due to the intrinsic instability of the epoxide ring (Pace-Asciak and Lee, 1989; Pace-Asciak and Asotra, 1989). The epoxide ring is rapidly hydrolysed *in vivo* by epoxide hydrolases to form the tri-hydroxy derivative, $TrXA_3$ (8,11,12-trihydroxy-eicosatrienoic acid) (Pace-Asciak and Lee, 1989; Pace-Asciak *et al.*, 1986). Similar reaction also occurs in acidic media, particularly during the acidic extraction of lipids. The inhibition of epoxide hydrolase by trichloropropene oxide (TCPO) (Pace-Asciak *et al.*, 1986) diverts the conversion of HxA_3 towards conjugation. Purified Yb2 subunits of glutathione transferases produce a glutathione conjugate upon reaction with HxA_3 (Laneuville *et al.*, 1991).

1.6 Biological role of 12/15 lipoxygenases:

1.6.1 Cell differentiation

15-LOX was discovered in rabbit reticulocytes as the agent responsible for the destruction of mitochondria during its maturation to erythrocytes. It was hypothesised that peroxidation of lipids leads to membrane destruction (Rapoport *et al.*, 1982; Grullich *et al.*, 2001). Recent evidence has shown that the enzyme may actually oligomerise and integrate into the lipid bilayer leading to the formation of hydrophilic pores (van Leyen *et al.*, 1998). 12/15-LOX

deficient mice, however, did not exhibit any significant defects in erythropoiesis (Sun and Funk, 1996). These mice did not exhibit any major malfunctions even under conditions of experimental anemia. Eye lens cells undergo a similar process of enucleation and destruction of organelles during maturation (van Leyen *et al.*, 1998). 15-LOX has been shown to play an important role in this process by preferentially targetting the organelle membranes. These reports also suggest that similar mechanism might be operative in keratinocytes. A role for 15-LOX in differentiation of human tracheobronchial cells has also been suggested (Hill *et al.*, 1998b). Retinoic acid induced differentiation and the expression of 15-LOX was observed in the later stages of the process, while undifferentiated cells do not produce any 15-LOX. 12/15-LOX translocated to the plasma membrane in monocytes incubated with apoptotic cells to the sites where actual contact was observed and altered the actin polymerization suggesting a role for this enzyme in the process of phagocytosis (Miller *et al.*, 2001). However, 12/15-LOX deficient mice did not exhibit any physical abnormalities such as defective eye lens or any immune abnormalities. The underlying mechanisms need to be elucidated.

1.6.2 Bronchial diseases

Metabolites of arachidonic acid may play a fundamental role as mediators in the development of airway inflammation in asthmatics (Chavis *et al.*, 1998). Since the identification of "slow reacting substance of anaphylaxis" as leukotrienes C_4/D_4 most of the research on asthma was concentrated around the key enzyme 5-lipoxygenase, which led to development of numerous drugs. However, a real breakthrough has not yet been achieved. It is now known that the bronchial hyperreactivity is not solely caused by increased release of mediators of bronchoconstriction and inflammation, but the increased sensitivity of bronchial receptors towards these mediators plays a pivotal role in the pathogenesis of asthma. The lung epithelial cells express 5- and 15- lipoxygenases, which produce a variety of metabolites, such as 5-HETE, 15-HETE and leukotrienes, but also cyclooxygenases (Salari and Chan-Yeung, 1989; Hill *et al.*, 1998a). The expression of these enzymes is tightly regulated in different types of cells (Moore *et al.*, 2001; Conrad *et al.*, 1992). Expression of 15-LOX enzyme is significantly increased in bronchial epithelial cells, and eosinophils (Conrad *et al.*, 1992; Levy *et al.*, 1993; Conrad and Lu, 2000). Consequently, large amounts of 15-HETE were observed in bronchial epithelial cells of asthmatic and emphysema patients as compared with those of normal subjects (Campbell *et al.*, 1993). Since 15-LOX dioxygenates not only free arachidonic acid, but also biomembrane phospholipids, a considerable alteration in the affinity of various receptors with respect to 15-HETE and other lipid mediators may be expected. IL-4, which is also upregulated during asthma promotes incorporation of 15-HETE into the phospholipids

(Profita *et al.*, 1999). Similarly exposure of human tracheal epithelial cells to ozone, a major oxidant in environmental pollution, increases 15-HETE production and its esterification into phospholipids (Alpert and Walenga, 1995). Early allergic response was enhanced upon pre-inhaling 15-HETE with no effect on the late response (Lai *et al.*, 1990b). Inhaled 15-HETE as such did not have differential effects on the airway of either normal or asthmatic individuals (Lai *et al.*, 1990a).

A number of reports have suggested a role for 15-LOX in other bronchial diseases like bronchitis and chronic obstructive pulmonary disease (COPD). In COPD patients, alveolar apoptosis was observed along with decrease in 15-LOX protein and vascular endothelial growth factor RNA levels (Kasahara *et al.*, 2000). Recently, increased 15-LOX-1 protein and mRNA expression was observed in the bronchial biopsies from patients suffering from chronic bronchitis (Zhu *et al.*, 2002). Further, increased IL-4 protein and mRNA levels were also associated with this condition. The association of IL-4 and 15-LOX-1 in bronchial diseases is interesting as it has been previously observed that IL-4 strongly upregulated 15-LOX-1 levels in bronchial cells (Conrad *et al.*, 1992).

1.6.3 Inflammation

12/15-lipoxygenase and its metabolites seem to exhibit both pro-inflammatory and anti-inflammatory activities. Elevated levels of 15-HETE and 13-HODE are associated with inflammation in various disorders such as human proctocolitis (Donoiwitz, 1985; Zijlstra *et al.*, 1991), psoriasis (Duell *et al.*, 1988). Psoriasis is an interesting example of the complexity of the lipoxygenase family. Both 12/15-LOX and 12R-LOX have been identified in psoriatic scales from patients (Boeglin *et al.*, 1998). Increased levels of 15(S)-HETE, 12(S)-HETE, 13(S)-HODE and 12(R)-HETE have been observed in psoriatic cells when compared to normal cells (Baer *et al.*, 1991). The relevance of these metabolites in the progression of the disease is unclear though pro-inflammatory cytokines such as IL-4 may play an important role in this disease (Asadullah *et al.*, 2002). Human rheumatoid arthritis B synoviocytes express 15-LOX-1 and produce 15-HETE (Liagre *et al.*, 1999). The role of this metabolite has not been studied in disease progression even though IL-4 levels were shown to be elevated under these conditions. 13-HODE, 12-HETE induced chemotaxis in neutrophils (Henricks *et al.*, 1991; Cunninghham *et al.*, 1986), however, 15-HETE inhibited the migration of these cells across cytokine activated endothelium (Takata *et al.*, 1994a).

The 12/15-LOX products are capable of exhibiting anti-inflammatory properties in a protective role or reverse the inflammatory symptoms. Leukotriene B4, a major 5-LOX product is one of the most important mediators of acute inflammation. Remodelling of

polymorphonuclear leukocyte membranes with 15-HETE reduced the leukotriene B4 cell surface receptor affinity (Takata *et al.*, 1994b), similarly increased levels of 15-HETE were correlated with inhibition of leukotriene B4 formation and synovial cell proliferation (Herlin *et al.*, 1990) in experimental arthritis. Leukotriene B4 synthesis and its chemotactic activity were specifically antagonised by 15-HETE in rat experimental glomulonephritis (Fischer *et al.*, 1992). Experimental glomerulonephritis was prevented in rat kidneys transfected with human 15-LOX-1 gene indicating a major anti-inflammatory role for 12/15-LOX (Munger *et al.*, 1999). 15-HETE inhibited superoxide formation and exocytosis in neutrophils stimulated with phorbol ester or platelet activating factor (Smith *et al.*, 1993).

1.6.4 Carcinogenesis

Many contradictory reports have appeared with respect to the role of 12/15 lipoxygenases in carcinogenesis. Transfection of PC3 prostate cancer cell line with 15-LOX-1 showed a pro-carcinogenic effect as measured by several parameters (Kelavkar *et al.*, 2001). These cells exhibited decreased anchorage dependent growth, increased proliferation and an increase in levels of angiogenic factors such as vascular endothelial growth factor. The cells also produced larger and more tumors when transplanted into athymic nude mice as compared to control cells. Prostatectomy specimens showed a significant co-expression of 12/15-LOX and mutant p53 coupled with the fact that elevated levels of 13-HODE were found in prostate carcinoma samples (Kelavkar *et al.*, 2000a; Spindler *et al.*, 1997). These data also correlated with elevated Gleason rating and the level of 12/15-LOX expression. A report which appeared six months later, however, showed that 15-LOX-2 metabolite 15-HETE caused dose dependent inhibition of PC3 proliferation by activating PPARγ transcription factors (Shappell *et al.*, 2001). Recently, Hsi *et al.*, 2002, observed that 13-HODE (15-LOX-1) and 15-HETE (15-LOX-2) have opposing effects on the regulation of PPARγ in epidermal growth factor signalling in prostate cancer cells and offer this as an explanation to the contradictory results obtained. Thrombin produced rapid pseudopod formation and detachment involving the formation of 12 and 15-HETE in rat prostatic carcinoma cells (Ross *et al.*, 2000).

Similar contrasting results were obtained in studies performed on colon carcinoma. Increased expression of 12/15-LOX was observed in colon carcinoma tissue as compared to matched normal tissue as measured by PCR, protein expression and immunohistochemistry by Ikawa *et al.*, 1999. On the other hand, Shureiqi *et al.*, 1999, found significantly reduced amounts of immunohistochemical staining and 13-HODE levels in colon carcinoma tissue as compared to normal ones. The same group also reported that non-steroidal anti inflammatory drugs (NSAIDs) could upregulate 15-LOX-1 and 13-HODE and induce apoptosis in these cells

(Shureiqi *et al.*, 2000). Caco-2 colon carcinoma cells exhibited apoptosis upon sodium butyrate treatment which corresponded to increased 12/15-LOX expression (Kamitani *et al.*, 1998, 2001).

Honn and group have shown in a number of reports that 12-HETE and platelet type 12-LOX can function as inducers of carcinogenesis and metastasis (Nie *et al.*, 2001; Honn *et al.*, 1994a). Furthermore, lipoxygenase inhibitor NDGA induces apoptosis in a number of carcinoma cell lines. The role of 12/15 lipoxygenase and its metabolites is complex and the mechanism of action of these contradictory effects is yet unclear.

1.6.5 Atherogenesis

Atherosclerotic lesions develop in a characteristic fashion, first appearing as fatty streaks. They consist of lipid-rich macrophages, foam cells localised beneath an intact endothelial cell layer in the arteries (Ross, 1999). Oxidation of LDL plays a key role in the early pathogenic events, leading to foam-cell formation and fatty streaks (Witzum, 1994; Steinberg and Witzum, 1999). Products of oxidized LDL (oxLDL) are chemotactic for monocytes, promote endothelial cell binding of monocytes, and once the monocytes have been recruited, inhibit the motility of macrophages. Macrophages can both initiate the oxidation of LDL and take up oxLDL in an unregulated manner, leading to foam-cell formation (Steinberg and Witzum, 1999; Cushing *et al.*, 1990; Watson *et al.*, 1997). There is now considerable evidence to support the presence of oxLDL *in vivo*, at least in animal models (Steinberg and Witzum, 1999). Many enzyme systems or nonenzymatic oxidative mechanisms have been demonstrated to induce oxidation *in vitro* (Heinecke, 1997). *In vitro* 15-lipoxygenase has been demonstrated to oxidise LDL (Kühn *et al.*, 1994a). 15-LOX transfected fibroblasts and monocytic cells exhibited increased conversion to the atherogenic form of LDL (Benz *et al.*, 1995) and impaired oxidation ability was observed in zymosan treated macrophages from 12/15-LOX deficient mice (Sun and Funk, 1996). Several other lines of evidence support the pro-atherogenic role of 12/15-LOX. The mechanism(s) responsible for oxidation of LDL *in vivo*, however, remain to be defined. Human atherosclerotic lesions showed 12/15-LOX activity (Henriksson *et al.*, 1985). Furthermore, 12/15-LOX mRNA and protein have also been detected in many different animal models (Hiltunen *et al.*, 1995; Yla-Herttuala *et al.*, 1990; Hugou *et al.*, 1995), however, due to non-specificity of the experimental procedures these results need to be confirmed. The oxLDL from cholesterol fed rabbits showed the presence of 12/15-LOX specific products and the presence correlated temporally with the onset of lipid deposition on the arterial wall (Kühn *et al.*, 1994b). Though similar results were obtained in human atherosclerotic lesions, the share of 12/15-LOX product is much reduced

(Folcik *et al.*, 1995; Kühn *et al.*, 1997). Added to this is the clarification of the exact enzymatic nature of the oxLDL formation as several other enzymatic systems such as cytochrome P450, myeloperoxidase and cyclooxyneases and non-enzymatic process can confuse the results.

Contrasting data showing the anti-atherogenic effects also exist. 15-LOX transgenic rabbits developed significantly lesser amount of atherosclerotic lesions when fed western type diet (Shen *et al.*, 1996). Experimental anaemia in rabbits causes the overexpression of 12/15-LOX and these animals fed on cholestrol rich diet developed fewer lesions as compared to control animals (Trebus *et al.*, 2002). Similar effects were also seen in mice (Paul *et al.*, 1999). Atherosclerosis is a complex disease requiring the interplay of several factors and the elucidation of the role of 12/15-LOX in this process requires intensive research.

1.6.6 Diabetes

Another area in which the role of 12/15-LOXs have been studied is diabetes melitus. In type I insulin dependent diabetes, the insulin producing β islet cells in the pancreas are destroyed by a cytokine dependent autoimmune process. The cytokines primarily involved are IL-1β, IFNγ and TNFα and according to current hypothesis, this is achieved *via* the activation of nitric oxide (NO) (Eizirik and Mandrup-Poulsen, 2001). IL-1β upregulates the expression of 12/15-LOX in rat insulinoma cell lines and in freshly isolated islet β cells (Bleich *et al.*, 1995). Increase in the 12/15-LOX mRNA, protein and 12-HETE were observed post IL-1β treatment. Some authors have, however, argued that this increase in 12-HETE production is due to increased substrate availability facilitated by NO mediated pathways rather than an upregulation of the enzyme activity as such (Ma *et al.*, 1996). 12/15-LOX deficient mice have been observed to be highly resistant to streptozotocin induced diabetes (Bleich *et al.*, 1999) and increase in 12-HETE levels were observed in patients suffering from this disease (Antonpillai *et al.*, 1996). 12/15-LOXs are lipid peroxidising enzymes and have the ability to generate free radical mediated oxidative stress. Rat insulinoma cells, Rin m5F, transfected with cGPx earlier demonstrated as an antagonist of 12/15-LOX, show increased resistance to cytokine induced apoptosis and necrosis (Lortz *et al.*, 2000). Cells transfected with other antioxidant enzymes such as, superoxide dismutase and catalase also show similar resistance (Tiedge *et al.*, 1999).

A different picture emerges of the role of 12/15-LOX in type-II, non-insulin dependent, diabetes. Hepoxilin induces the production of insulin when injected in rats (Pace-Asciak *et al.*, 1999) and 12-HETE also induces the production of glucagon from the α cells (Falck *et*

al., 1983). Thus, the exact role of 12/15-LOX in both types of diabetes needs to be elucidated, preferably by the use of specific inhibitors.

1.7 Apoptosis

Apoptosis, or programmed cell death, is considered a normal physiological process and a major form of cell death that is used to remove damaged or infected cells throughout the life. Apoptosis is also a mechanism by which the organism deals with stress, injury and factors threatening its integrity such as infection. Apoptosis is therefore important in normal cell development, occurring during embryogenesis as well as in the maintenance of tissue homeostasis (Nagata, 1997).

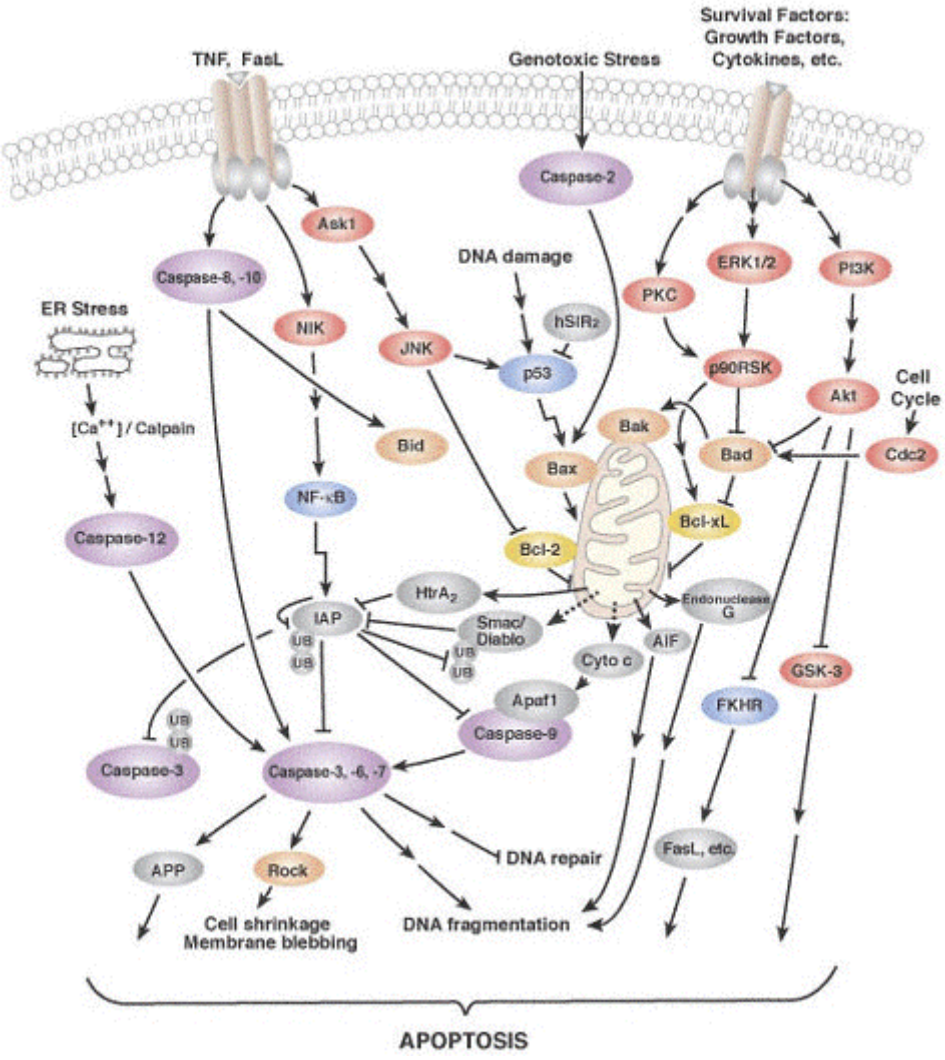


Figure 6. Overview of apoptotic signalling pathways.

1.7.1 General mechanisms of apoptosis:

Apoptosis is a regulated physiological process leading to cell death characterized by cell shrinkage, membrane blebbing and DNA fragmentation (Kerr *et al.*, 1972). Caspases, a family of cysteine proteases, are central elements of apoptosis. Initiator caspases (including 8, 9, 10 and 12) are closely coupled to pro-apototic signals. Once activated, these caspases cleave and activate downstream effector caspases (including 3, 6 and 7) which in turn cleave cytoskeletal and nuclear proteins and induce apoptosis (Thornberry and Lazebnik, 1998). Principally two pathways initiate the signalling cascade that results in apoptosis.

1.7.1.1 Intrinsic pathway: This pathway is triggered in response to DNA damage, oxidative stress, chemotherapeutic agents and other types of stress (Vaux, 2002). It involves the Bcl-2 family of proteins which consists of pro- and anti-apoptotic members (Gross *et al.*, 1999). The anti-apoptotic members such as Bcl-2 and Bcl-X_L exist in the mitochondria (Kluck *et al.*, 1997; Vander Heiden *et al.*, 1997). Upon receiving the stress signal, the pro-apoptotic memebrs (bax, bid, bak, PUMA) translocate to the mitochondria and neutralise the anti-apoptotic members by oligomerisation (Shimizu *et al.*, 1999; Rosse *et al.*, 1998; Luo *et al.*, 1998). This results in the permeabilisation of the membrane resulting in the release of apoptogenic factors such as SMAC/Diablo, cytochrome c, SIMPs and AIF (Garl and Rudin, 1998; Matsuyama and Reed, 2000). Cytochrome c binds to APAF-1 to form the apoptosome resulting in the cleavage of caspase-9 and progression of the cascade *via* the effector caspases (Li *et al.*, 1997).

A number of pro-apoptotic stimuli including FasL, TNF, DNA damage and ER stress can initiate this pathway (Budihardjo *et al.*, 1999; Nagakawa, 1999). Cytosolic p53, induced by DNA damage, has been observed to activate bax, PUMA, Noxa mitochondrial translocation (Oda *et al.*, 2000). This process can be inhibited by the overexpression of Bcl-2 and Bcl-X_L (Camilleri-Broet *et al.*, 2000). Fas ligand or TNF receptors can also induce apoptosis utilising both intrinsic and extrinsic pathway (Scaffidi *et al.*, 1998). The caspase-8 activated by these signals cleaves Bid which then translocates to the mitochondria to bind to Bcl-X_L triggering cytochrome c release (Li *et al.*, 1998). ER stress leads to the calcium-mediated activation of caspase 12 and proceeds *via* the cleavage of caspase-3 (Nagakawa, 1999). Anti-apoptotic ligands including growth factors and cytokines activate AKT kinase and p90RSK, which inhibit Bad and prevent cytochrome c release (Bonni *et al.*, 1999; Datta *et al.*, 1999). TNFR can also stimulate an anti-apoptotic pathway by inducing IAP, which directly inhibits

caspases 3, 7 and 9 (Roy *et al.*, 1997). AIF (apoptosis inducing factor) released from the mitochondria (like cytochrome c) can directly bind to the nuclear DNA and induce its damage leading to cell death (van Loo *et al.*, 2002).

1.7.1.2 Extrinsic pathway: This pathway is used in a number important physiological functions such as cytotoxic T cell mediated apoptosis. External signals such as FasL (produced by cytotoxic T cells on their surface) and TNFα can activate pathway specific receptors of the TNF superfamily known as death receptors (TNF-R1, CD95, TRAIL R1 and R2) (Baker and Reddy, 1998; Ashkenazi and Dixit, 1998). The receptor oligomerisation results in the recruitment of specific adapter proteins such as FADD, TRADD, RIP and DAXX (Chinnaiyan *et al.*, 1995; Hsu *et al.*, 1995; Yang *et al.*, 1997; Chaudhary *et al.*, 1997). This complex is designated as DISC (death- inducing signalling complex). The formation of DISC leads to the recruitment and cleavage of pro-caspase-8 and 10 to their active forms. Caspase-8 and 10 function as initiator caspases leading to the activation of effector caspases. Cells can be divided into type I and type II depending upon the pathway utilised by activated caspase-8 (Scaffidi *et al.*, 1998). In type I cells, sufficient amounts of caspase-8 exists and therefore directly cleave the effector caspases. While the type II, as described earlier, due to limited amounts of caspase-8, the mitochondrial amplification step is utilised.

1.7.2 Potential role of 12/15-LOX in apoptosis:

Arachidonic acid and its lipoxygenase and cyclooxygenase products have been observed to modulate apoptosis signalling (Tang *et al.*, 2002). This occurs either by various metabolites of the AA cascade acting as ligands for signalling pathways or *via* the generation of oxidative stress by lipid hydroperoxides. 12/15 lipoxygenases can act on both free fatty acid and on membrane phopholipids to produce lipid hydroperoxides. In rat glioma cells, it has been observed that glutathione depletion leads to increased production of 12/15-LOX and causes cell death (Higuchi and Yoshimoto, 2002). NSAIDs induce the expression of 15-LOX-1 in colorectal and gastric cancer cells and trigger apoptosis ((Shureiqi *et al.*, 2000; Wu *et al.*, 2003). SC-236, a specific inhibitor of COX-2 induced 15-LOX-1 and 13-HODE production (not 15-HETE) and initiated apoptosis in gastric cancer cells (Wu *et al.*, 2003). Apoptosis was abolished by the inhibition of 15-LOX-1. Sodium butyrate treatment of rat intestinal epithelial cells led to differentiation and apoptosis, induction of 12/15-LOX has been suggested to be involved in this process (Kamitani *et al.*, 1999). Treatment of RBL2H3 cells with 2-deoxyglucose leads to the production of lipid hydroperxides and subsequent apoptosis.

Overexpression of the mitochondrial form of PHGPx, a scavenger of lipid hydroperoxide radical, led to the reduction in hydroperoxide levels and suppression of apoptosis (Imai *et al.*, 1996; Nomura *et al.*, 1999). Similar protection against apoptosis by PHGPx was observed in rabbit aortic smooth muscle cells challenged with linoleic acid hydroperoxide (Brigelius-Flóhe *et al.*, 2000).

Increased levels of 12-HETE, produced by the reduction of 12-HpETE by glutathione peroxidase has been observed in several cancers and is correlated to their metastaic potential (Tang and Honn, 1999). Overexpression of 12-LOX platelet type enzyme in MCF-7 mammary carcinoma cells, in human prostate adenocarcinoma cells resulted in cell proliferation and larger tumors (Liu *et al.*, 1996; Connolly and Rose, 1998). Inhibition of 12-LOX platelet type enzyme by NDGA and other inhibitors in Walker 256 carcinoma cells, gastric cancer cells and in lewis lung carcinoma cells resulted in the growth inhibition and apoptosis (Wong *et al.*, 2001; Tang *et al.*, 1997; Honn *et al.*, 1994b). Similar effect has also been observed in melanoma and prostate cancer cells (Onoda *et al.*, 1994; Pidgeon *et al.*, 2002). Thus, the two 12 lipoxygenase isoforms have opposite effects on cell proliferation and apoptosis. It could be argued that conversion of 12-HpETE to 12-HETE reduces the oxidative stress in the cell. Another possibility is that 12-HETE functions as a ligand for proliferative pathways. AA and its products act as ligands for PPAR and other receptors. These receptors could have variable effects on cell proliferation.

1.7.3 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor gene family and are important regulators of fatty acid metabolism. PPARs act in a similar manner to other nuclear hormone receptors (Kersten *et al.*, 2000). First, they bind a specific element in the promoter region of target genes. PPAR bind to the promoter as a heterodimer with retinoid X receptor (RXR) (Gearing *et al.*, 1993). Second, they activate transcription in response to the binding of ligand. Eicosanoids, fatty acids and synthetic compounds known as peroxisome proliferators act as ligands to PPARs.

Three PPAR isotypes have been identified: α,β and γ. Some ligands are shared by the three isotypes, such as polyunsaturated fatty acids and probably oxidized fatty acids. Several compounds bind with high affinity to PPARα, including long-chain unsaturated fatty acids such as linoleic acid, branched, conjugated and oxidized fatty acids such as phytanic acid and conjugated linolenic acid, and eicosanoids such as 8(S)-HETE and leukotriene (LT) B_4. The

prostaglandin 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is the most potent natural ligand of PPARγ. 12/15-LOX products 13-HODE and 15-HETE also act as ligands for this receptor (Kersten *et al.*, 2000; Gearing v 1993; Hihi *et al.*, 2002; Forman *et al.*, 1995; Lehmann *et al.*, 1995). Much of the function of PPARs can be extrapolated from the identity of their target genes, which so far all belong to pathways of lipid transport and metabolism.

The role of 12/15-LOX and PPARγ in atherosclerosis is quite confusing. PPARγ activation in monocytes and macropphages induce their apoptosis (Chinetti *et al.*, 1998) and PPARγ liagnds reduce the development of atherosclerotic lesions in LDL-receptor knockout mice indicating anti-atherosclerotic effect (Li *et al.*, 2000). While, pro-atherosclerotic effect was the induction of CD36, a scavenger receptor of oxLDL by IL-4 and 12/15-LOX induced PPARγ activation (Huang *et al.*, 1999). This was clearly demonstrated by the lack of CD36 induction in 12/15-LOX deficient mice and in PPARγ deficient stem cells (Chawla *et al.*, 2001).

PPARγ activation has also been implicated in lung diseases. Increased PPARγ activation was observed in asthmatic patients as compared to normal controls and was correlated to apoptosis, airway remodelling and inflammation (Benayoun *et al.*, 2001). Further, PPARγ agonists induced apoptosis in human lung carcinoma cells (Theocharis *et al.*, 2002; Satoh *et al.*, 2002; Inoue *et al.*, 2001; Tsubouchi *et al.*, 2000). Activation of death receptors, specifically TRAIL and inhibition of anti-apoptotic subunit of NF-κB p65/RelA have been implicated in PPARγ induced apoptosis, especially in macrophages (Chinetti *et al.*, 1998; Ji *et al.*, 2001).

2. Aim and objectives of the study

Lipid mediators such as, leukotrienes, prostaglandins, lipoxins and lipoxygenase metabolites are important constituents of inflammatory process. Of these, 12/15-lipoxygenase and its metabolites were observed prominently in bronchial inflammatory diseases like asthma and bronchitis. Th2 cytokines, IL-4 and IL-13, have been observed to induce the expression of the 12/15-LOX and are also present in bronchial inflammatory conditions. The 12/15-LOX metabolites, especially 15-HETE and 13-HODE, have been observed to function as ligands for nuclear receptor/transcription factor PPARγ. The exact nature of the influence these factors have in the pathogenesis of inflammatory bronchial diseases is not clear. The enzyme has also been implicated in other disorders such as insulin dependent diabetes. In rat β islet cells, novel metabolites of 12/15-LOX, hepoxilins, have been observed though the pathways involved in their biosynthesis are little understood.

I. The aim of the study was to characterise the mechanism of induction of 12/15-LOX expression by IL-4 in lung carcinoma cell line, A549. Experiments were aimed at elucidating the signal transduction pathways involved. Our main focus was on understanding changes occurring in the chromatin structure, especially histone modifications and the interaction between the various transcription factors and co-activators required for the induction of mRNA synthesis.

II. One of the objectives of the study was to determine the role of 12/15-LOX enzyme and its metabolites in the apoptotic process, or cell death observed during bronchial inflammation. In particular, the nature of involvement of PPARγ and the characterisation of the downstream signalling steps leading to apoptosis.

III. The nature of the biosynthetic pathway of hepoxilin was investigated in rat β islet cells. The intrinsic hepoxilin synthase activity of 12/15-LOX was examined along with the regulation of synthesis of hepoxilins.

3. EXPERIMENTAL PROCEDURES

3.1 MATERIALS

FATTY ACIDS AND EICOSANOIDS

Arachidonic acid, linoleic acid, 12-HpETE, 12-HETE, 15-HETE, 13-HODE - Cayman (USA).

CULTURE REAGENTS

Fetal Calf Serum (FCS), Dulbecco's Modified Eagles Medium (DMEM), Trypsin/EDTA, Streptomycin/Penicillin – Seromed (Germany); Yeast Nitrogen Base, Yeast extract, Tryptone, Peptone, Agar– Difco (USA).

SEPARATION MEDIA

Agarose – Roth (Germany); Silica gel-60 TLC plates – Merck (Germany); Rotiphorese acrylamide Gel 30 – ROTH (Germany); Supesil silica HPLC column – Supelco (Germany), Novapak C_{18} RPLC column – VDS Optilab (Germany).

KITS

RNeasy Mini Kit and QIAshredder - Qiagen (Germany); Protein Determination kit – Biorad (USA); Chemiluminiscent system - Santa Cruz (Germany); Luciferase Reporter Assay – Promega (Germany); PolyFect Transfection Kit- Qiagen (Germany); Lipofectamine 2000 – Invitrogen (Germany); QuikChange kit – Stratagene (USA); Cell Death detection ELISA – Roche (Germany).

ANTIBODIES

Actin – Sigma (Germany); PPARγ – Cayman (USA); histone H3 and acetyl histone H3 – Upstate, Germany; Bcl-X_L – Calbiochem (Germany); Pan acetyl, STAT6, cytochrome c, porin VDAC, Protein A agarose, anti-mouse, anti-rabbit, anti-goat secondary antibodies conjugated with horse radish peroxidase – Santa Cruz (Germany).

RT-PCR REAGENTS

Expand reverse transcriptase – Roche Biochemicals (Germany), Taq DNA Polymerase – AB Technologies (Germany), Pfu Turbo Polymerase – Stratagene (USA), Platinum Taq Polymerase – Invitrogen (Germany).

CASPASE SUBSTRATE AND INHIBITOR

Caspase-3 substrate Ac-DEVD-pNA and inhibitor Z-VAD-FMK – Calbiochem (Germany).

ENZYMES

Proteinase K- Roche Biochemicals (Germany); T4 DNA ligase-Gene Craft (Germany); Terminal deoxynucleotide transferase (TdT)- Pharmacia (Sweden); Restriction enzymes were obtained from NEB (USA).

RADIOCHEMICALS

^{14}C-Arachidonic acid (55 mCi/mmol); ^{3}H-Acetyl Coenzyme-A - Amersham (England).

PRIMERS

All primers were purchased from TIB Biomol (Germany).

SEQUENCING

Automated fluorescent DNA sequencing was performed by SeqLab (Germany).

OTHER REAGENTS

All organic solvents used in HPLC and GC-MS experiments were of LiChrosolv or SupraSolv quality, all other reagents were of analytical grade and were mainly obtained from Sigma Chemicals (Germany).

3.2 Media and Buffers

LB MEDIUM

10 g/L	Tryptone,
5 g/L	Yeast extract,
5 g/L	NaCl.

The pH was adjusted to 7.2 and autoclaved.

1x TBE

10.8 g/L	Tris base
5.5 g/L	Boric acid
4 ml	EDTA (0.5 M)

3.3 Mammalian cell culture

A549 cells (DSMZ, Braunschweig, Germany) and HeLa (DSMZ, Braunschweiz, Germany) were cultured in Dulbecco's Modified Eagle's medium supplemented with 10 % fetal calf serum. Normal human bronchial epithelial cells (BEAS-2B) (ATCC, Washington D.C., USA) were cultivated in a modified LHC-9 medium with 0.5 ng/ml recombinant epidermal growth factor (EGF), 500 ng/ml hydrocortisone, 0.005 mg/ml insulin, 0.035 mg/ml bovine pituitary extract, 500 nM ethanolamine, 500 nM phosphoethanolamine, 0.01 mg/ml transferrin, 6.5 ng/ml 3,3',5-triiodothyronine, 500 ng/ml epinephrine, 0.1 ng/ml retinoic acid and trace elements as supplied by Clonetics Corporation, USA. Rinm5F, rat insulinoma cells (ATCC, Washington D.C., USA), was cultured in RPMI 1640 medium supplemented with 10 % fetal calf serum. The cells were grown in humidified incubators at 37 ^{0}C with 5 % CO_2.

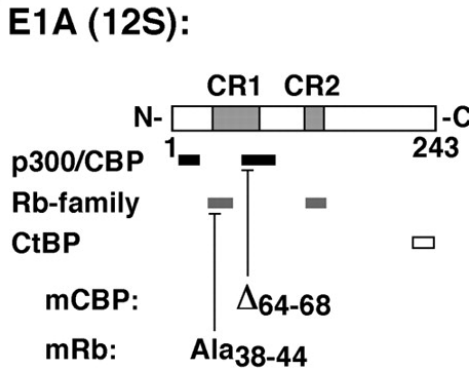
3.3.1 Transient and stable transfection of cell lines

Transient transfection of DNA was performed in A549 and HeLa cells using Polyfect transfection reagent (Qiagen, Germany). 5x10^5 cells were seeded into 35 mm tissue culture plates. Next morning, 1.5 µg of plasmid DNA was mixed with 12 µl of Polyfect reagent in 100 µl antibiotic free medium and DNA-liposome complexes were allowed to form at room temperature for 20 minutes. The complexes were gently seeded onto the cells and incubated at normal conditions for 24 h. The cells were used for further experiments at the end of this period. For Rinm5F cells, Lipofectamine 2000 reagent was utilised along with 1 µg of DNA. The cells were cotransfected with 0.1 µg of control plasmid pRSVLACZ to normalise for transfection efficiency by performing β-galactosidase assay.

Stably transfected cells were achieved by transferring transiently transfected cells into medium containing restrictive antibiotic G418 (Invitrogen, Germany). For HeLa cells 800 µg/ml and for Rinm5F cells 700 µg/ml of G418 was used. Individual colonies of G418 cells were separately grown for at least 1 month in the antibiotic. After this period, the cells were transferred and maintained in medium containing half the selective concentration of the antibiotic. The clones expressing highest amount of foreign protein as evidenced from Western blotting were used for further experiments. Sodium selenite (Sigma, Germany) 10 nM was supplemented into the medium in the case of Rinm5F cells transfected with cGPx or PHGPx. Rinm5F stably transfected with cytosolic glutathione peroxidase (cGPx), kind gift from Dr. Tiedge, Hannover, Germany, were cultures in the same culture medium as above supplemented with 350 µg/ml G418 (Invitrogen) and 10 nM sodium selenite.

3.4 Plasmids

Plasmids wt E1A, mCBP and mRb were kind gifts of Dr. A. Hecht, Freiburg, Germany. wt E1A consists of 2 domains which bind to CBP and Rb proteins and inhibit their actions. Plasmid mCBP is the E1A protein from which the CBP binding domain has been mutated and in the mRb protein the Rb binding domain has been mutated (Hecht *et al.*, 2000).



PPARγ dominant negative plasmid was a kind gift from Prof. V.K.K. Chattejee, Oxford University, UK. The plasmid contained the complete PPARγ tagged with FLAG in which leu^{468} was mutated to Ala and Glu^{471} was mutated to Ala (Adams *et al.*, 1997). The mutant protein retains the ligand and DNA binding activity but has no transactivating activity because of impaired binding to the coactivators like CBP and steroid receptor coactivator-1.

FADD dominant negative vector was a kind gift from Dr. M.L. Schmitz, Heidelberg, Gemany. In this plasmid death effector domain (N terminal 80 amino acids) truncated FADD protein was expressed which even though could bind to the death receptors, could not propagate the signal by its inability to bind to caspase-8 (Hofmann *et al.*, 2001).

PHGPx overexpression vector was a kind gift from Prof. R. Brigelius-Flóhe, Potsdam, Germany. The complete PHGPx gene along with the regulatory 3'UTR was cloned into pcDNA3 (Brigelius-Flóhe *et al.*, 1997).

Other plasmid were constructed in the laboratory by cloning required fragments and all the fragments and the cloning procedure was confirmed by DNA sequencing (SeqLab, Germany).

Peroxisome prolifator response element (PPRE) was PCR amplified and cloned in pGL3 basic vector (Promega) at the Kpn 1 and Hind III sites.

12/15-LOX was amplified from rat cDNA by PCR and cloned into mammalian expression vector pcDNA3 (Invitrogen) at the BamHI and XhoI sites and into pET15b bacterial expression vector (Novagen) at the Nde I and Xho I sites.

3.5 Western blotting

3.5.1 Preparation of total protein from cells

Cells were lysed directly on the plates by the addition of protein lysis buffer containing 50 mM Pipes/HCl (pH 6.5), 2 mM EDTA, 0.1% Chaps, 20 µg/ml Leupeptin, 10 µg/ml Pepstatin A,10 µg/ml Aprotinin , 5 mM DTT and 1 mM PMSF (Cell Technologies, USA). The cells were scraped and lysed by freezing and thawing 3 times. The mixture was centrifuged at 10,000xg at 4 ^{0}C for 15 minutes to remove the cell debris. Protein concentration was determined using a modified Lowry‘s method (BioRad, germany).

3.5.2 SDS-PolyAcrylamide Gel Electrophoresis and Immunoblotting

10% SDS Polyacrylamide gel was prepared according to the following recipe. For the separating gel of 10 ml volume: 4 ml of distilled water, 2.5 of 1.5 M Tris-HCl (pH 8.8), 3.3 ml of 30% Polyacrylamide mix (37.5:1 ratio of mono and bis acrylamide) (Roth, Germany), 100 µl of 10% SDS, 100 µl 10% ammonium persulphate (APS) and 15 µl N,N, N’,N’ tetramethylethylenediamine (TEMED). For 5 ml of stacking gel: 3.4 ml of distilled water, 630 µl of 1 M Tris-HCl (pH 6.8), 830 µl 30% Polyacrylamide mix, 50 µl 10% SDS, 50 µl 10% APS and 5 µl TEMED. The gel was cast in MiniProtean III apparatus (BioRad, Germany). The protein samples were mixed with SDS sample solution (Rotiload, Roth, Germany) and boiled for 5 minutes for complete denaturation. This mixture was loaded onto the gel and electrophoresed Immunoblotting was performed utilising the semi dry transfer method. Nitrocellulose membrane (Schleisser and Schuell, Germany) and 6 layers of Whatman 3 paper were cut exactly to the size of the gel and soaked in transfer buffer along with the gel. A stack was made with 3 sheets on the top and bottom of the gel and membrane and placed in between the graphite plates of the transfer apparatus (Biometra, Germany). Transfer was performed at 0.8 mA/cm^2 current for 1 hour. The efficiency of the transfer was confirmed by staining the membrane with a 0.1% solution (containing 1% acetic acid) of Ponceau S (Sigma, Germany). The excess stain was washed with water. After visualisation of the protein, the blot was completely washed off the stain with water and put into blocking solution (5% nonfat milk powder in PBS containing 0.05% Tween 20) for 1 h at room temperature. The blots were then incubated in the blocking solution containing 1:500 – 1:1000 dilution of the primary antibody for 1h at RT. After washing in wash solution (PBS containing 0.05% Tween 20) for 10 minutes, the blot was incubated with the secondary antibody (against the species of the primary antibody and linked to horse radish peroxidase)

(1:5000 dilution) for 1 h at RT. After 3 washes of 5 minutes each with wash buffer, the specific bands were developed using Chemiluminiscent system (Santa Cruz, Germany). Equal volumes of solution A and B were mixed and the blot was incubated in this solution for 1 minute. The blot was immediately exposed to an X-Ray film in dark for varying periods of time ranging from 5 sec to 2 min and automatically developed. The specific bands were scanned and quantified densitometrically using TINA version 2.09g. All immunoblots, were sequentially incubated with anti-β-actin as control, and specific signals adjusted in relation to the expression of this house-keeping gene.

3.6 Cell fractionation

3.6.1 Nuclear extract preparation

Cells were washed with ice cold PBS, scraped and pelleted at 10,000xg, 4 ^{0}C. The pellet was resuspended in 400µl of hypotonic buffer (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl) and incubated on ice for 15 minutes and simultaneously lysed with the addition of 100µl of 2.5% NP40. Nuclei were pelleted for 1 minute at 2000xg. The nuclear pellet was resuspended in protein lysis buffer (Dignam *et al.*, 1983).

3.6.2 Preparation of mitochondria

Cells were washed twice with PBS, trypsinised and the cell pellet was collected. The pellet was resuspended in 5 volumes of homogenisation buffer (20 mM HEPES 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 250 mM Sucrose) and homogenised for 5 minutes using a tight fitting dounce homogeniser. The whole procedure was carried out at 4 ^{0}C. The effectivity of the procedure was checked by trypan blue exclusion test. Nuclei and cell debris were pelleted at 2,000xg. Mitochondria were pelleted by centrifugation at 10,000xg for 30 minutes at 4 ^{0}C. The pellet was resuspended in protein lysis buffer (3.5.1). The supernatant was further centrifuged at 100,000xg for 45 minutes at 4 ^{0}C. The supernatant was used as the cytosolic extract. Protein concentration was determined both in the mitochondrial and cytosolic fractions. Equal amounts of protein were used for further experiments.

3.7 Immunoprecipitation

Immunoprecipitaions were performed by incubating the protein extracts with 2 µg of the primary antibody for 1 hour at 4 ^{0}C. The immune complexes were incubated with protein-A agarose (Santa Cruz) for 1 hour at 4 ^{0}C. The beads were spun down (2500xg, 2 min, 4^{0}C),

washed 3 times with RIPA buffer (1% nonidet P40, 0.5% deoxycholate, and 0.1% SDS in PBS) and the immune complex released by boiling in SDS sample solution and electrophoresed. The proteins were eluted from the beads with elution buffer (1% SDS in 0.1 M $NaHCO_3$) when subsequent immunoprecipitations or reactions were to be performed.

3.8 Chromatin immunoprecipitaion

Formaldehyde was added to the cells at a final concentration of 1% and incubated for 20 minutes at room temperature. The reaction was stopped by the addition of glycine to a final concentration of 0.125M. The cells were washed with cold PBS and harvested. The nuclear extract was prepared according to Dignam *et al.*, 1983 and sonicated at maximal power for 30 seconds twice to shear the genomic DNA. Immunoprecipitations were performed with various antibodies. Crosslinking was reversed in the immunoprecipitated complexes by the addition of NaCl to a final concentration of 200 mM and incubation at 65 ^{0}C for 6 hours. The DNA was purified by proteinase K treatment (150µg/ml) for 1 h followed by phenol/chloroform extraction and precipitation by ethanol. The presence of specific promoter were detected by PCR with specifc primers. The extract aliquoted prior to the immunoprecipitaions was used to prepare control input genomic DNA, which was also used for PCR analysis. For Western blotting, protein was directly denatured by electrophoresis sample buffer and applied to SDS-PAGE.

3.9 RNA preparation

Total RNA was prepared using RNAsy kit fom Qiagen, Germany. Cells were washed with PBS 3 times and lysed in 350 µl RNA lysis buffer and sonicated at maximum wattage for 30 seconds. The lysate was mixed with equal volumes of 70 % ethanol and loaded onto the column. The column was spun at maximum speed for 15 seconds at RT. The column was washed with RW1 and PR1 buffer and RNA was eluted in 30 µl Rnase free water by centrifugation and stored at –80 ^{0}C.

3.10 Reverse transription and polymaerase chain reaction

Reverse transcription was performed using 5 µg of RNA which was denatured at 65 ^{0}C for 10 minutes and to which reaction buffer, 1,5 mM $MgCl_2$,1 mM dNTPs, 20 mM DTT, 50 pmoles of $(dT)_{18}$ primer and 1 µl of AMV reverse transciptase (Roche, Germany) was added. The reaction was performed at 42 ^{0}C for 1h. 1/20th of the resultant cDNA was used in the subsequent PCR reaction. PCR was performed with 5 pmoles of forward and reverse primer,

200 µM of each dNTP and 1 U of Taq polymerase. For quantitative PCR, 25 cycles were used and for normal PCR 35 cycles were used. β-actin primers were used to perform control reactions to quantitate the product formed. Genomic PCR was performed using 10 ng of genomic DNA as template. Negative and positive controls were used with each set of PCR reaction. The primers and reaction conditions are listed below.

STAT6 element of 15-LOX-1 gene:
5‘ GTGGGGTGGTGGGGGGTGAAAG 3‘
5‘ CTCCTCACCTCTCATCCCACTGC 3‘
94 ^{0}C 3min; 94 ^{0}C 30 s, 55 ^{0}C 30 s, 72 ^{0}C 30 s for 35 cycles; 72 ^{0}C for 7 min, 4 ^{0}C.

15-LOX-1 promoter (1 kb):
5‘ AAGCTAATTCACTCTGGTGGGGTGG 3‘
5‘ AAGATGTTTCGCTCCTTCTGGAGG 3’
94 ^{0}C 3min; 94 ^{0}C 30 s, 55 ^{0}C 30 s, 72 ^{0}C 1 min for 35 cycles; 72 ^{0}C for 7 min, 4 ^{0}C.

Human Peroxisomal Fatty Acyl Coenzyme A Oxidase Gene containing PPRE:
5‘ CGGAAGCTTCGCGACGACCAGCTGGC 3‘
5‘ GGCAGATCTTACGTTGACGTGAGGTCGG 3‘
94 ^{0}C 3min; 94 ^{0}C 30 s, 60 ^{0}C 30 s, 72 ^{0}C 2.5 min for 35 cycles; 72 ^{0}C for 7 min, 4 ^{0}C.

Rat 12/15 lipoxygenase gene:
5’ CGACATATGTGTCTACCGCATCCGC 3’
5’ TGGCTCGAGTCAGATGGCCACGCTGTT 3’
94 ^{0}C 3 min; 94 ^{0}C 1 min, 55 ^{0}C 1 min, 72 ^{0}C 2 min, 35 cycles; 72 ^{0}C 7 min; 4 ^{0}C.

15-LOX-1 cDNA :
5' GGGGCTGGCCGACCTCGCTATC 3'
5' TCCTGTGCGGGGCAGCTGGAGC 3'
94 ^{0}C 3 min; 94 ^{0}C 1 min, 72 ^{0}C 1 min, 35 cycles; 72 ^{0}C 7 min; 4 ^{0}C

The PCR mixture was mixed with DNA loading buffer and electrophoresed in 2% agarose gel in 1x TBE at 7 volts/cm of gel. DNA was visualised under UV after staining the gel with 0.5 µg/ml of ethidium bromide.

3.11 Electrophoretic Mobility Shift Assay (EMSA)

Double stranded oligonucleotide containing the transcription factor binding element was used as probe in the gel shift assays. The assays were performed with nuclear extracts. The oligonucleotide was made double stranded by heating the mixture of the two strands at 94 ^{0}C for 5 minutes and then was allowed to cool down slowly to RT. The probe was labelled with γ ^{32}P ATP (sp. activity 3000 Ci/mmol) using T4 polynucleotide kinase at 37 ^{0}C for 30 minutes. Protein was incubated with 30,000 cpm of labelled oligonucleotide in a buffer containing 10 µM Tris pH 7.5; 1 mM EDTA; 100 mM NaCl; 5 % glycerol along with 1 µg of Poly dIdC

(Roche, Germany) for 20 minutes at RT in the presence or absence of various competing oligonucleotide sequnces (Schreiber *et al.*, 1989). The reaction mixture was electrophoresed on a 6% PAGE in 1xTBE and visualised by autoradiography.

3.12 Reporter assay

3.12.1 Luciferase assay

The cells were washed twice with ice-cold PBS and lysed with 100 µl luciferase lysis buffer (Promega, Germany). The lysis was completed by freezing and thawing 3 times and after centrifugation the supernatant was utilised for assay of luciferase and β-galactosidase. Luciferase assay was performed by vortexing 5 µl of the extract with 50 µl of luciferase assay solution (Promega, germany) and the bioluminescence was measured by scintillation counting.

3.12.2 β-galactosidase assay

For quantification of β-galactosidase activity, 50 µl of the extract was incubated at 37^0C for 10 min, then 100 µl of pre-warmed 4 mg/ml o-nitrophenyl para-D-galactopyranoside (ONPG) in 0.1 M sodium phosphate buffer (pH 7.5) was added. After a suitable time of reaction, 300 µl of 1 M Na_2CO_3 was added, and the absorbance at 420 nm of the solution was measured with a spectrophotometer. The β-galactosidase activity was expressed as ONPG unit (1 unit: the activity producing 1 mmol o-nitrophenol per minute, normalised against the inoculated cell number). The β-galactosidase activity was determined as the average value from triplicate dishes under the same transfection conditions.

3.13 DNA affinity chromatography

The specified DNA was amplified by PCR. 100 pmoles of this DNA was end labelled with biotin16-dUTP using terminal deoxynucleotide transferase (TdT) enzyme (Pharmacia, Germany). The end labelled DNA was purified by ethanol precipitation and bound to streptavidin-coated magnetic beads (Roche, Germany). 100 µl of the beads were washed 3 times with 100 µl of DNA binding buffer (10 µM Tris pH 7.5; 1 mM EDTA; 100 mM NaCl) and each time the beads were collected using magnetic particle collector (Dynabeads, Sweden). The biotin labelled DNA was mixed with the bead with at least 2 volumes of DNA binding buffer. The mixture was kept at 4 ^{O}C with vigorous shaking for 10 minutes. The magnetic particles were collected and washed 3 times with DNA wash buffer (10 µM Tris pH 7.5; 1 mM EDTA; 1 M NaCl). The beads were further washed 3 times with 100 µl of protein

binding buffer (10 μM Tris pH 7.5; 1 mM EDTA; 100 mM NaCl; 5 % glycerol). The beads were incubated with 100 μg of protein in protein binding buffer containing 10 μg of Poly dIdC. The incubation was performed under continuous shaking at 4 ^{0}C for 1h. The beads were once again collected and washed 3 times with protein binding buffer. The specifically bound proteins were eluted using protein binding buffer containing 2 M NaCl. The solution was desalted by overnight dialysis against PBS at 4 ^{0}C and electrophoresed on SDS-PAGE.

3.14 *In vitro* histone acetyltransferase (HAT) assay

Filter binding assays were performed as described (Ogryzko *et al.*, 1996) with minor modifications. 3.3 mg/ml of histones (Sigma) were acetylated in a reaction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 % glycerol, 1mM PMSF. (^{3}H) acetyl CoA (Amersham, UK) and 6 μg of protein extract for 30-60 minutes at 30 ^{0}C. The reaction mixture was spotted onto P81 phosphocellulose paper (Upstate Biotechnology, USA) and washed for 30 minutes with 0.2 M carbonate buffer (pH 9.2). The filter paper was dried and used for liquid scintillation counting. Similar experiments were performed using non-radioactive acetyl-CoA.. The reaction mixture was denatured and loaded onto a SDS PAGE. The Western blot was probed with anti-acetylhistone H3 antibodies and developed using Chemiluminiscent detection system.

3.15 Assays for apoptosis

3.15.1 Cell Death ELISA

Cell Death Detection ELISA kit (Roche Biochemicals, Germany) was also used to quantify the apoptosis in cells. The cells were lysed by a hypotonic solution and the cytoplasmic extract was incubated with anti-histone and anti-DNA antibodies. The complexes bound to the ELISA plate were quantified using horse raddish peroxidase enzyme and DTNB reagent. 10^4 cells were plated on 96-well plates in DMEM medium. Effectors or inhibitors of apoptosis were added, and apoptotic cell death was determined after 72 hours with above mentioned ELISA kit.

3.15.2 Annexin-V assay

For the annexin-V assay, cells grown on glass coverslips were washed with PBS and incubated for 15 minutes at room temperature with a solution of annexin-V fluos and propidium iodide (Roche Biochemicals, Germany). Cells were then washed twice with PBS and observed under a fluorescence microscope. Normal cells do not get stained at all, while

apoptotic cells are stained green (annexin-V fluos) and necrotic cells red (propidium iodide). Fluorescence microscopy was performed with a BX-40 Olympus microscope witha mercury lamp, appropriate filter set and automatic photomicrography attachment. A minimum of 200 cells were counted.

3.15.3 TUNEL assay

TUNEL assay was performed by washing paraformaldehyde-fixed (4% paraformaldehyde in PBS for 30 minutes at RT) cells on coverslip 3 times with PBS and then permeabilized using 0.5% saponin at room temperature for 30 min. After washing with TdT buffer cells were incubated with 0.5 µM biotin dUTP, 150 U /ml TdT in 30 µl TdT buffer (Roche Biochemicals, Germany) in a humidified chamber at 37 ^{0}C for 30 minutes. After washing twice with PBS, the cells were incubated with 1:1000 solution in PBS of streptavidin-conjugated horse radish peroxidase (GIBCO, Germany) for 10 minutes at room temperature. Coverslips were then washed for 30 minutes with 3 washes of PBS. The colour was developed with True Blue (KPL Labs, Germany) peroxidase substrate, and coverslips were observed under a light microscope. Apoptotic cells were stained blue.

3.15.4 Caspase-3 assay

Caspase-3 assay was performed using Ac-DEVD-pNA as substrate. Cells were lysed with lysis buffer (50 mM HEPES, pH7.4, 100 mM NaCl, 0.1 % CHAPS, 1 mM DTT, 100 µM EDTA) and centrifuged at 10,000xg for 10 min. 100 µg of protein were incubated with 200 µM solution of Ac-DEVD-pNA (Calbiochem, Germany) in reaction buffer (lysis buffer with 10 mM DTT and 10% glycerol) at 37 ^{0}C. The development of yellow colour at 405 nm indicated caspase-3 activity. The reaction was monitored periodically for 3-4 hours. The rate of reaction was calculated as difference in the absorbance at 405 nm per unit time. The results were represented as fold increase in caspase-3 activity over the control reactions. Z-VAD-FMK (Calbiochem, Germany) was used as inhibitor of caspase-3 at a concentration of 100 µM an hour prior to standard treatment.

3.16 Detection of PPARγ ligand

Labelled fatty acids as ligands to PPARγ were detected by immunoprecipitating the PPARγ in pre-treated cells and then detecting the fatty acid attached to it by radio-TLC. Cells (1x 10^{6}) were incubated with 0.25 µCi of [^{14}C]-arachidonic acid [sp. act. 55 mCi/mmol] (Amersham, Germany) for 24 hours. After the incorporation of the radioactive arachidonic acid (AA), cells

were washed and treated according to experimental set-up. Cell lysate was prepared and immunoprecipitated with anti-PPARγ antibody in 1 ml RIPA buffer for 1 hour at 4 ^{0}C. The immune complexes were allowed to bind to protein A agarose (Santa Cruz) for 1 hour. The beads were spun down, washed 3 times with RIPA buffer and resuspended in 200 µl of PBS. This solution was acidified with HCl to pH 3.5 and lipids were extracted 3 times with ethyl acetate. After drying under nitrogen stream the sample was reconstituted in ethyl acetate and loaded onto a Silica TLC plate (Merck, Germany) and developed with hexane:ether:acetic acid (50: 50 : 0.1 v/v) as solvent system. For quantitation, the TLC plate was scanned on radio-TLC scanner (Berthold Instruments, Germany). AA, 5-, 12- and 15-HETEs and various prostaglandins were run on the side as standards.

3.17 High Performance Liquid Chromatography analysis (HPLC)

Purified proteins or cell extracts in PBS were incubated with arachidonic acid (100µM) the reaction was allowed to proceed at 37 ^{0}C for 20 minutes. Reduction of hydroperoxy fatty acids to their corresponding hydroxy derivatives was achieved by the addition of a molar excess of sodium borohydride. The reaction mixture was acidified to pH 3.5 and lipids were extracted with an equal volume of ethyl acetate. Straight phase HPLC analysis was performed on a Supelco-SIL column (250 x 4.6 mm, 5 µm) using n-hexane: 2-propanol: acetic acid (100:2:0.1, by vol.) as mobile phase at a flow rate of 1 ml/min. reverse phase HPLC was performed on a Novapak C_{18} reverse phase column and 80 : 20, acteonitrile : water as the running buffer. Conjugated dienes were detected and quantified at 235 nm using a diode array detector (Shimadzu).

3.18 Assay of hepoxilin A_3 synthase activity

The hepoxilin synthase A_3 activity was measured by incubating the purified protein or cell lysate with 20 µM 12(S)-HpETE in a volume of 200 µl for 30 minutes at 37 ^{O}C and measuring the formation of hepoxilins using GC-MS or HPLC. The reaction was stopped by acidification with 1N HCl to pH 3.0 The reaction mixture was allowed to incubate at room temperature in acidic conditions for 15 minutes before extracting the lipids twice with 3 volumes of ethyl acetate. The lipid fractions were extracted with ethyl acetate, evaporated to dryness under nitrogen, reconstituted in n-Hexane and analysed with GC-MS.

3.18.1 Gas chromatography-Mass spectrometry (GC-MS)

The lipids were converted to their methyl esters by the addition of 300 µl ethereal diazomethane for 5 min. Thereafter the samples were evaporated to dryness, 30µl N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) were added and the mixture was derivatised by heating at 60^{o}C for 30 min. Gas chromatography-mass spectrometry (GC-MS) was performed by means of a Varian Saturn 4D GC-MS-MS system equipped with a Supelco DB5-MS column (30 m x 0.25 mm; 0.25 µM d_f). The temperature program was started at 150^{o}C increasing to 250^{o}C within 10 min with a rate of 10^{o}C/min. The temp. of injector and transfer line were 230^{o}C and 220^{o}C respectively.

3.18.2 Detection of hepoxilin A_3 by HPLC

Protein was incubated with 100 µM AA at 37^{O}C for 30 minutes. The reaction was stopped and the lipids were extracted by the addition of ether-methanol-1M citrate (135:15:1, v/v). The organic phase was evaporated and the residue was taken into 100 µl ethyl acetate and derivatised by the addition of 9-Anthryldiazomethane (100 µg/reaction) (Reynaud *et al.*, 1994). The reaction mixture was stirred at room temperature for one hour. The ethyl acetate was then evaporated, the residue was reconstituted in the solvent system and the lipids were loaded onto a Novapk C_{18} (250 x 46 mm; 5 µm particle size) reverse phase column. Acetonitrile-methanol-water (90:6:4 v/v) was used as mobile phase. The eluate was uv-monitored using a Shimadzu diode-array detector set at 254 nm and fluorescence-monitored (excitation 254 nm, emission 400nm) using a Shimadzu fluorescence detector.

3.19 Cloning of rat 12/15-LOX

Total RNA was extracted from Rinm5F cells using RNeasy kit and reverse transcribed using AMV reverse transcriptase enzyme. The reaction was performed at 42 ^{0}C for 45 minutes using a dT_{18} primer. Complete 12/15-LOX gene was amplified with Pfu Polymerase and 200 µM dNTPs using primers as mentioned above. A small aliquot of the reaction was used for instant PCR cloning using TOPO TA cloning kit. The ligation was performed for 5 minutes and then E. coli DH5α was transformed with 2 µl of the ligation reaction using heat shock. The mixture was plated onto LB ampicillin plates and the white colonies were grown in 3 ml LB (80 µg/ml ampicillin), plasmid prepared and were screened for the presence of the insert using restriction enzymes. The positive clones were sequenced from both directions using M13 forward and reverse primers and automated fluorescent sequencing. The entire sequence

was compared with the published rat 12/15-LOX sequence (Accession number. NM_031010) using the Blast server (www.ncbi.nlm.nih.gov/Blast).

3.20 Cloning and expression of rat 12/15-LOX in bacteria

The 12/15-LOX gene insert was cloned into the Nde I and Xho I sites in PET 15b vector (Novagen). The positive colonies were screened and purified. For overexpression experiments, *E. coli* BL21 DE3 cells were transformed with the plasmid. A 3 ml overnight culture was established from a single colony. Next morning, a 10 ml culture was cultivated in LB (80µg/ml ampicillin and 37 µg/ml chloramphenicol). The cells were grown till O.D. 0.6 and then the recombinant protein production was induced by the addition of 1 µM IPTG for 3 h at 37 ^{0}C. The bacteria was pelleted by centrifugation and resuspended in PBS. Lysis was performed by sonication. The lysate was used for all further experiments.

3.20 Statisics

All experiments were repeated at least three times and the representative pictures have been shown. The data were presented as mean ± SD. Statistical comparisons between groups were made using Student's t test for paired observations. Significance was achieved at the $p<0.05$ level. All calculations were performed at www. graphpad.com.

4. Results

4.1 Transcriptional Regulation of 15-LOX-1 expression

4.1.1 Induction of 15-LOX-1 expression in A549 human lung epithelial cells

A549 cells were cultured for various time periods in the presence of 670 pM IL-4. The cells were harvested and the lysates were analysed for the expression of 15-LOX-1 mRNA by RT-PCR using 15-LOX-1 specific primers. 15-LOX-1 mRNA was first observed after minimum 12 h of IL-4 stimulation. The highest mRNA concentration was detected after a 24 h incubation period (Fig. 7A). After longer incubation periods the mRNA levels dropped perceptibly. To find out whether IL-4 has to be present during the entire incubation period or whether a single cytokine stimulus may be sufficient to induce 15-LOX-1 expression the following experiment was carried out. A549 cells were exposed to IL-4 for various time periods, the cytokine was washed away and incubation was resumed for a total of 24 h. Finally, the expression of 15-LOX-1 mRNA was analysed by RT-PCR.

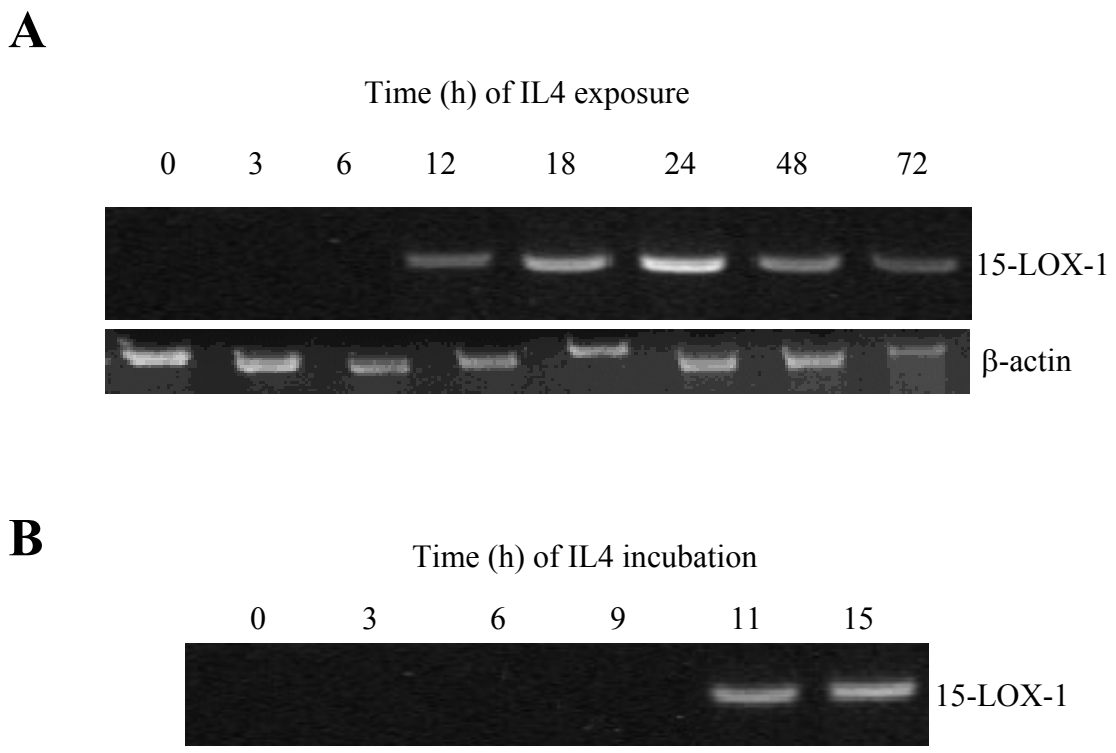


Figure 7. Induction of 15-LOX-1 mRNA was delayed and requires continuous exposure to IL-4. Panel A: A549 cells were incubated with 670 pM IL-4 in serum-free medium for different periods (0-72 h), the cells were harvested, and total RNA was extracted. Semiquantitative RT-PCR was performed to detect 15-LOX-1. β-Actin RT-PCR was used for the normalization of 15-LOX-1 expression. **Panel B:** A549 cells were exposed to IL-4 (670 pM) for varying periods (0-11 h). IL-4 was removed by washing thrice with PBS, and incubation was resumed for a total of 24 h; then the cells were lysed for RNA extraction. 15-LOX-1 expression was assayed by RT-PCR.

As shown in Fig. 7B, 15-LOX-1 expression in A549 cells required a minimum of 11 hours continuous exposure to IL-4. These data indicate that a single IL-4 stimulus was not sufficient to up-regulate expression of the 15-LOX-1 mRNA.

4.1.2 Activation of 15-LOX-1 mRNA expression does not require de novo protein synthesis

As a time lag of 12 h was observed in the start of transcription, it was probable that *de novo* synthesis of additional regulatory factors was required. To test this hypothesis stimulation with IL-4 was performed in the presence of cycloheximide (10 µg/ml), a protein synthesis inhibitor and the presence of 15-LOX-1 mRNA detected by PCR. As shown in Fig. 8. cycloheximide did not affect the activation of 15-LOX-1

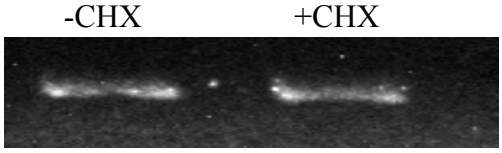


Figure 8. *De novo* protein synthesis was not required for induction of 15-LOX-1 mRNA. Cells were exposed to cycloheximide (10 µg/ml) along with IL-4 (670 pM) for 12 h. 15-LOX-1 expression was assayed by RT-PCR.

4.1.3 IL-4 upregulates histone acetyltransferases in A549 cells

Activation of cellular acetyltransferases may constitute an additional regulatory element in the intracellular signal transduction cascade (Kouzarides, 2000). Acetylation of histones causes conformational changes of nuclear proteins leading to demasking of potential transcription factor binding sites, so that the transcription factor may bind to the promoter of target genes. Since histone acetylation has recently been implicated in the induction of 15-LOX-1 expression in CaCo-2 cells (Kamitani *et al.*, 2001), the effect of IL-4 on HAT activity was tested in A549 cells. For this purpose, cells were exposed to IL-4 (670 pM) for 3 h and the cell lysates were assayed for HAT activity. IL-4 significantly upregulated HAT activity even after a relativly short incubation periods (Fig. 9). The HAT activity is the sum of several catalytic processes and involves the activity of various proteins. One of these enzymes is the transactivating protein CBP/p300, which exhibits a strong HAT activity. To find out whether or not CBP/p300 was involved in IL-4 induced upregulation of acetyltransferase activity in

A549 cells, the cells were transfected with the viral oncoprotein wtE1A, which has been identified as an endogenous inhibitor of CBP/p300.

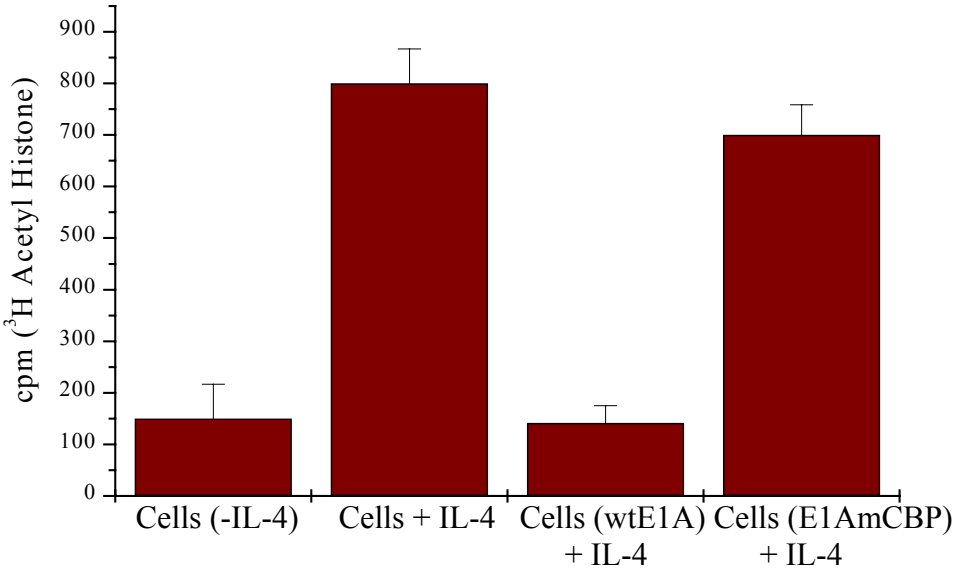


Figure 9. IL-4 uprgulates histone acetyl transferase activity via CBP/p300. A549 cells were exposed to IL-4 for 3 h, and cell lysates were used to measure the histone acetyltransferase activity. Cells were either untransfected or transfected with the wtE1A or with its antagonizing mutant E1AmCBP. The experiments were repeated at least thrice (n = 4).

After IL-4 treatment the transfected cells exhibited a significantly reduced HAT activity (Fig. 9). However, in cells transfected with E1AmCBP, a mutant of E1A protein incapable of binding to CBP/p300, no increase in IL-4 induced HAT activity was observed.. These data indicate that the augmented acetyltransferase activity was mainly due to activation of CBP/p300.

4.1.4 HDACs stimulate IL-4 induced 15-LOX-1 expression

The degree of acetylation of nuclear histones depends on cellular HAT activity but also on the activity state of histone deacetylases (HDACs). In resting cells there appears to be a steady state of acetylating and deacetylating events and inhibitors or activators of either process may shift the equilibrium in either direction. Recently, it has been reported that activation of HDAC may cause alterations in the chromatin state and may inhibit gene transcription (Kao *et al.*, 2000). If the hypothesis that CBP/p300 catalyzed histone acetylation is important for IL-4 induced 15-LOX-1 expression) was true, then inhibitors of cellular HDAC are likely to act synergistically to IL-4 or may even be capable of inducing 15-LOX-1 expression in the

absence of IL-4. To test this conclusion A549 cells were incubated with suboptimal doses (335 pM) of IL-4 in the presence of sodium butyrate (NaBT) and the expression of 15-LOX was assayed by Western blotting.

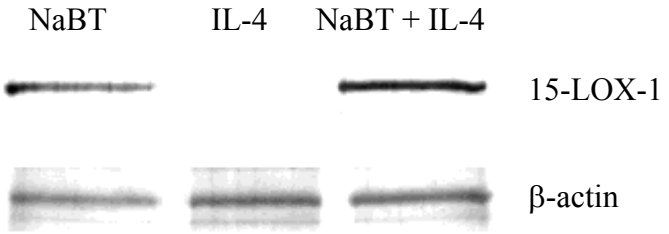


Figure 10. IL-4 synergestically upregulates the expression of 15-LOX-1expression by sodium butyrate. Cells were cultured for 24 h in the presence of a suboptimal dose of IL-4 (330 pM) alone, 2 mM sodium butyrate (NaBT) alone, or of both together. The cells lysates were then analyzed for 15-LOX-1 expression using a specific antibody. The amount of protein was normalized by Western blotting with β-actin antibody.

From Fig. 10. it can be seen that IL-4 at suboptimal concentrations did not induce 15-LOX-1 expression. However, in the presence of NaBT a strong LOX signal was observed. Interestingly, NaBT did also induce 15-LOX-1 expression in the absence of IL-4 albeit a weaker signal was detected. Similar results have recently been reported for other cellular systems (Kamitani *et al.*, 2001).

4.1.5 In vitro binding of transcription factors and the role of tyrosine phosphorylation

Since histone acetylation may be important for binding of transcription factors to the promoter of the 15-LOX-1 gene, *in vitro* transcription factor binding assays were carried out. A549 cells were incubated with IL-4 for different time periods. Nuclear extracts were prepared and binding studies of nuclear proteins to the 15-LOX-1 promoter were performed. In cells, which were cultured in the absence of IL-4, no promoter binding proteins were detected (Fig. 12, lane 1). In contrast, a variety of 15-LOX-1 promoter-binding proteins were present in the nucleus of IL-4-treated cells (Fig. 12, lane 2 and 3).

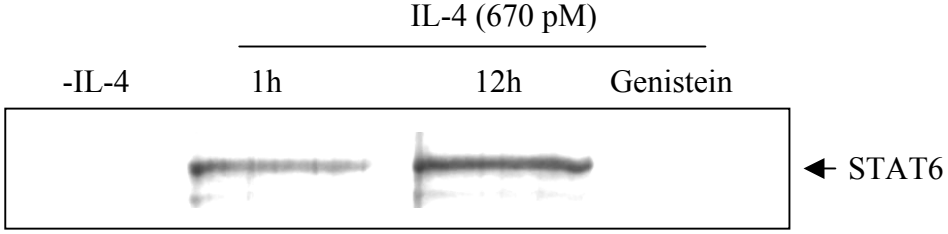


Figure 11. Tyrosine phosphorylation was essential for STAT6 activation. A549 cells were treated with IL-4 for 0, 1and 6 h or pretreated with genistein. Invitro binding assays were performed with the nuclear extracts and 15-LOX-1 promoter. The proteins bound to the promoter were assayed by Western blotting for the presence of STAT6.

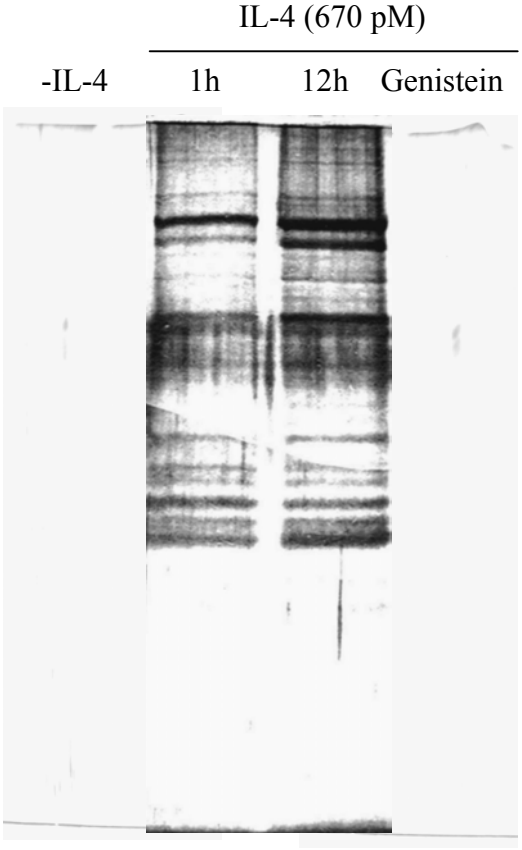


Figure 12. *In vitro* binding of transcription factors was dependent on tyrosine phosphorylation. A. A549 cells were cultured for 1 and 12 h in the absence or presence of IL-4 (670 pM). Before starting the incubation, one batch of cells was pretreated with genistein (25 µg/ml) for 15 min. After the incubation period, cells were harvested, nuclear extracts were prepared, and DNA binding assays were performed. *First lane*, cells incubated for 12 h in the absence of IL-4; *second lane*, cells incubated for 1 h in the presence of IL-4; *third lane*, cells incubated for 12 h in the presence of IL-4; *fourth lane*, genistein-pretreated cells incubated for 12 h in the presence of IL-4.

As expected STAT6 was one of the major components and its identity was confirmed by Western blots using commercially available anti-STAT6 antibodies (fig. 11). Interestingly, the pattern of the binding proteins was very similar when cells were treated with IL-4 for 1 or 12 hours (Fig. 11, lane 2 and 3). These data indicate that under *in vitro* conditions the transcription factors including STAT6 are capable of binding to the immobilized 15-LOX-1 promoter and that 1 h incubation was sufficient for maximal *in vitro* binding. Combining these data (rapid in vitro binding) with the results shown in Fig. 7 (delayed 15-LOX-1 expression) one may conclude that *in vivo* the binding of phosphorylated STAT6 to the promoter was hindered. Alternatively, co-activators exhibiting a prolonged time-dependence may be required for transcriptional regulation of the 15-LOX-1 gene.

It has been reported for other cell types that tyrosine phosphorylation was involved in IL-4 and IL-13 induced 15-LOX-1 expression (Conrad and Lu, 2000). Thus, the effect of genistein, a potent tyrosine kinase inhibitor, on protein phosphorylation and on the binding activity of nuclear proteins to the 15-LOX-1 promoter was examined. A549 cells were treated with genistein (25 µg/ml) for 30 min. After washing away the inhibitor 670 pM IL-4 was added and the cells were cultured for additional 12 hours. Subsequently, the nuclear extracts were analyzed for the presence of 15-LOX-1 promoter binding proteins. From Fig. 12 (lane 4) it can be seen that genistein completely blocked the binding of proteins to the promoter and abrogated 15-LOX-1 expression (Fig. 13). Similarly treated cells were also analysed for the presence of acetylated STAT6. Genistein was observed to inhibit IL-4 induced acetylation of STAT6 (Fig. 13).

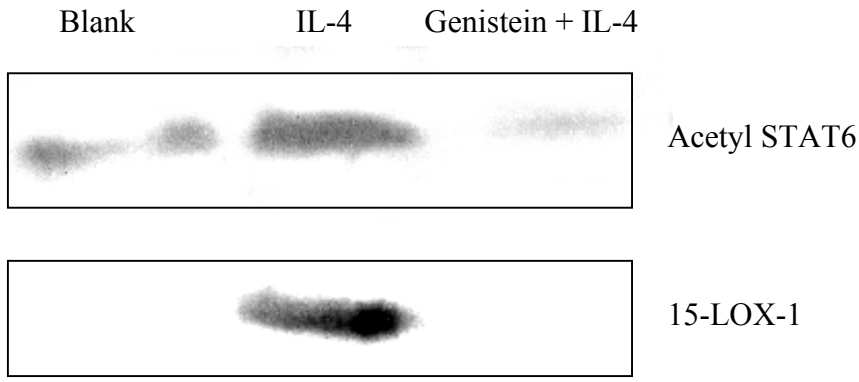


Figure 13. Tyrosine phosphorylation of STAT6 was important for 15-LOX-1 expression. Cells were treated with IL-4 for 12 h with or without preincubation with genistein. The proteins were assayed for acetyl STAT6 and 15-LOX-1 by Western blotting.

These data suggests that tyrosine phosphorylation in A549 cells was a prerequisite for STAT6 acetylation and binding to the 15-LOX-1 promoter.

4.1.6 In vivo binding of STAT6 and histones to the 15-LOX-1 promoter

From Fig. 12, it was concluded that under *in vitro* conditions phosphorylated STAT6 was capable of binding to the 15-LOX-1 promoter. The next series of experiments were aimed at addressing the question of whether or not such a binding may actually occur *in vivo*. For this purpose A549 cells were cultured in the presence of IL-4 for various time periods and DNA binding proteins were cross-linked to the nucleic acid by formaldehyde treatment. Then STAT6 was immunoprecipitated with a specific antibody and the cross-linked DNA was

analysed by PCR using 15-LOX-1 promoter specific primers. We found that the earliest binding of STAT6 was detected after 11 hours of IL-4 exposure (Fig. 14). These data were somewhat surprising since both STAT6 phosphorylation and its *in vitro* binding were rapid processes. Thus, it was concluded that the binding of STAT6 to the 15-LOX-1 promoter *in vivo* was hindered during early phases of the incubation period.

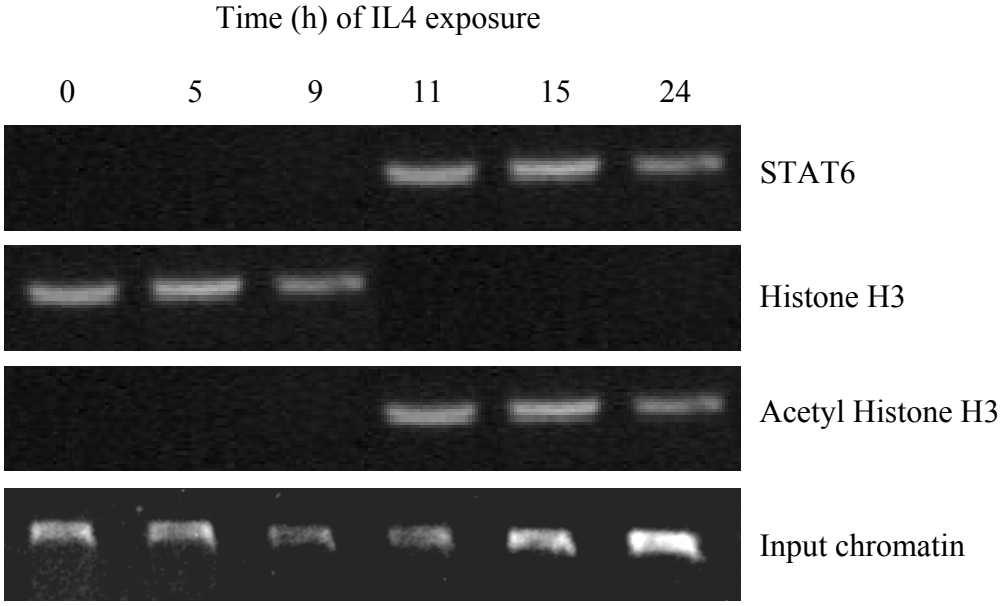


Figure 14. Differential kinetics of *in vivo* binding of STAT6, histone, and acetylated histone to the 15-LOX-1 promoter. A549 cells were exposed to IL-4 (670 pM) for the periods indicated and then treated with formaldehyde to cross-link DNA binding proteins to the DNA. The protein-nucleic acid complexes were immunoprecipitated with anti-STAT6, anti-histone H3, and anti-acetylhistone H3 antibodies. The cross-linked DNA was purified and analyzed by PCR for the presence of 15-LOX-1 promoter DNA. An aliquot of the complexes was removed before the immunoprecipitations and was similarly processed and used as a control for the PCR reaction**.** This DNA was referred to as input chromatin.

Similar *in vivo* binding studies were performed using anti-histone H3 (middle lane) and anti-acetylhistone H3 antibodies (lower lane). It was observed that non-acetylated histones were bound at the early phase of the induction process. In contrast, STAT6 and acetylated histones were bound exclusively at later stages. These data indicate an inverse correlation between the binding of non-acetylated histone and the activation of 15-LOX-1 gene.

4.1.7 STAT6 and histones physically interact in the acetylated form

Immunoprecipitations were performed to obtain experimental evidence for a physical interaction between STAT6 and histones upon IL4 stimulation. To check for in vivo interaction, the cells were treated with 1% formaldehyde to crosslink the proteins and then immunoprecipitation was performed on the protein extract and then a dual immunoprecipitation strategy was carried out. After the first immunoprecipitation with the anti-acetylhistone H3 antibody the protein was divided into 2 lots. The majority (75%) was used for the second round of immunoprecipitation with STAT6 antibody and western blotting using an anti-acetyl antibody as probe. This laborious method had to be applied since no antibody against acetyl STAT6 was currently available. For immunostaining of the acetylhistone H3 only 25% of the initial immunoprecipitate was used because of its abundance in the cell. This strategy as well as the different cross-reactivity of the anti-acetyl antibody with different acetylated proteins does not allow a direct comparison of the relative amounts of STAT6 and histones H3. At 11 hours after IL4 stimulation, acetyl STAT6 was bound to the acetyl histones while at 4 hours very little of this interaction was observed (Fig. 15).

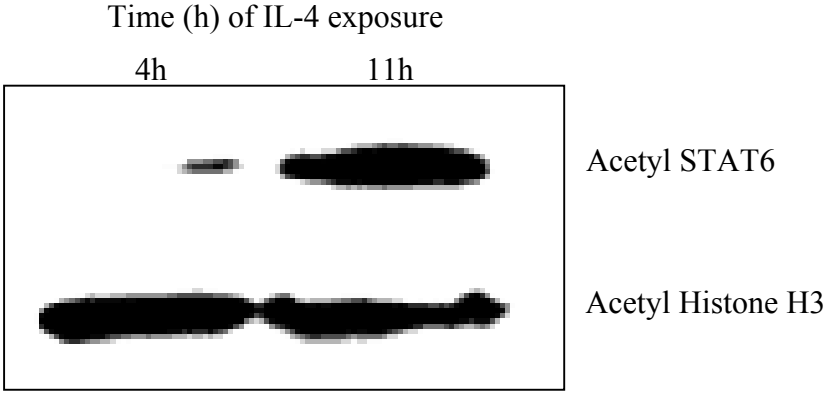


Figure 15. Interaction between acetyl STAT6 and acetyl histones. Cells were exposed to IL-4 for 4 and 11 h, and DNA-binding proteins were cross-linked to the DNA by formaldehyde treatment. Cross-linking was reversed and the proteins were analysed by Western blotting for acetylhistone H3 and acetyl STAT6.

This observation was in accordance with the data obtained from the chromatin immunoprecipitaion experiments (Fig. 14). Further, after the reversal of crosslinking, the same immunoprecipitaion was performed with STAT6 antibody and similar results were obtained. Here again, an increased binding of acetyl STAT6 to acetylhistones H3 during the time course of IL-4 treatment was observed (Fig. 16, left panel).

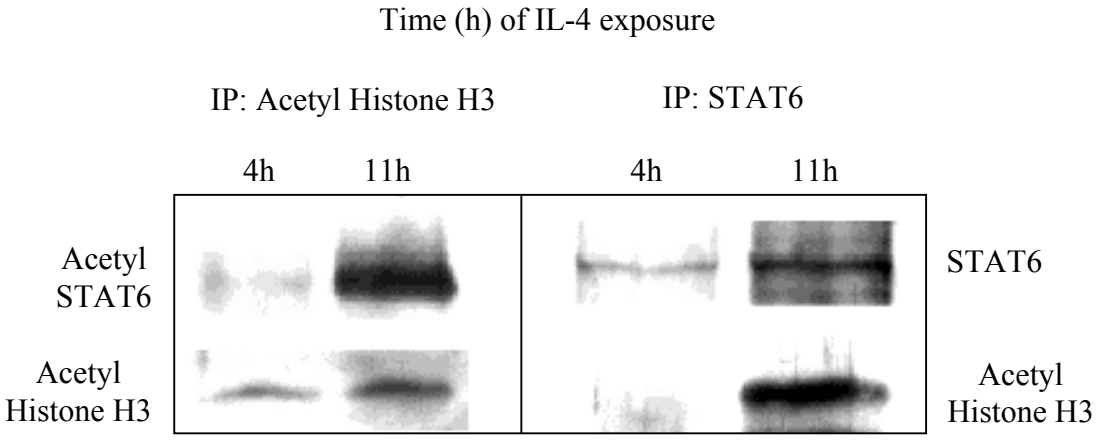


Figure 16. Kinetics of interaction between STAT6 and histone. Cells were incubated with IL-4 for 4 and 11 h, and proteins were cross-linked with formaldehyde. The proteins were then immunoprecipitated with either Acetyl Histone H3 or STAT6 antibodies. After the reversal of crosslinking, the proteins were analysed by Westrern blotting for STAT6, acetyl STAT6 and acetyl histone H3.

When immunoprecipitaion was carried out with a STAT6 antibody, no co-precipitation of acetylhistones H3 at 4 h of IL-4 exposure but a strong signal after 11 h was observed (Fig. 16, right panel). The chromatin immunoprecipitation experiments (Fig. 14) showed that after 11 hours of IL-4 exposure only acetylated histones H3 and acetylSTAT6 are bound at the 15 LOX-1 promoter and the increased binding of acetyl histone to STAT6 during the time course of IL-4 exposure (Fig. 15) was in line with these data.

4.1.8 CBP/p300 plays an important role in the acetylation of STAT6

If acetylation of STAT6 and of nuclear histones was crucial for IL-4 induced transcription of the 15-LOX-1 gene, inhibition of acetylation was expected to block this process. It is known from the literature that CBP/p300, a transactivating protein with HAT activity, is capable of acetylating STAT6 (McDonald and Reich, 1999). To study the role of CBP/p300, A549 cells were transfected with wtE1A and one E1A mutant, which differed from the wild-type with respect to its functional properties. The mutant E1AmCBP, which lacks amino acids 64-68 - the CBP binding region - acts as E1A antagonist and does not inhibit the acetyltransferase activity of CBP/p300 (Hecht *et al.*, 2000). After transient transfection of A549 cells with the appropriate cDNA constructs the cells were treated with IL-4 for 12 h. Cells were harvested, lysed and acetylation of STAT6 was measured by Western-blotting using an acetyl-specific antibody (Fig. 17).

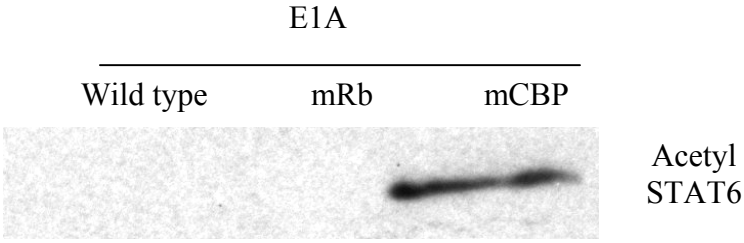


Figure 17. CBP/p300 acetylates STAT6. Cells were transfected with either wtE1A, E1AmRb or E1AmCBP vector. Cells were then treated with IL-4 for 12 h and protein was analysed by Western blot for acetyl STAT6 protein.

In cells transfected with wtE1A there was no enhancement in STAT6 acetylation. In contrast, cells transfected with the E1AmCBP mutant (E1A antagonist) showed a strong STAT6 acetylation signal.

4.1.9 Acetylation of STAT6 is essential for its promoter binding

The data shown in the previous figures indicate that STAT6 acetylation was upregulated when the cells are stimulated with IL-4 and that the acetyltransferase activity of CBP/p300 was involved. To find out whether STAT6 acetylation was required for its binding to the 15-LOX-1 promoter EMSA were carried out. Cells transfected with wtE1A and with the non-inhibitory mutant E1AmCBP were cultured in the presence of IL-4 for 12 h. The cells were then lysed and EMSA was carried out with STAT6 binding consensus sequences. In Fig. 18 it can be seen that STAT6 binding occurred when cells were treated with IL-4 and similar results were obtained when cells were transfected with the E1A antagonist E1AmCBP, which cannot bind CBP/p300 and therefore was unable to inhibit the CBP/p300 acetylase activity. On the other hand, no STAT6 binding was observed when acetylation was prevented by transfecting the cells with wtE1A.

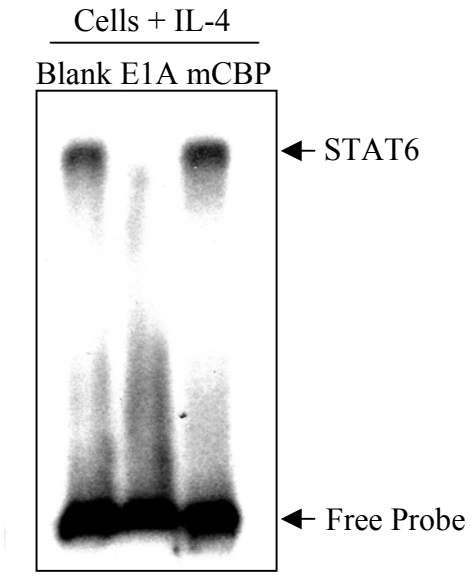


Figure 18. Acetylation of STAT6 was a prerequisite for its promoter binding. EMSA was performed with the protein extracts from the wtE1A and E1AmCBP transfected cells and STAT6 binding element obtained from the 15-LOX-1 promoter.

4.1.10 CBP/p300 is required for 15-LOX-1 expression

The experiments so far have pointed to the importance of CBP/p300 in IL-4 stimulated 15-LOX-1 expression. To confirm this effect, different amounts of wt E1A cDNA were transfected in A549 cells and the cells were stimulated with IL-4. The suppression of 15-LOX-1 expression was found to be dose-dependent by both immunoblotting (Fig. 19a) and activity assays (Fig. 19b).

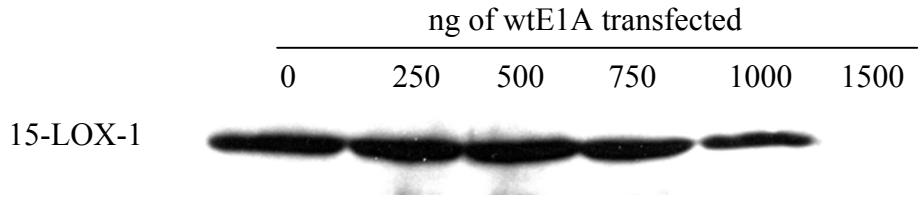


Figure 19a. CBP/p300 was essential for 15-LOX-1 expression. Dose dependent inhibition of 15-LOX-1 by wtE1A was performed by transfecting cells with varying amounts of wtE1A (0-1.5 µg) and probing the cell lysates with anti 15-LOX-1 antibody.

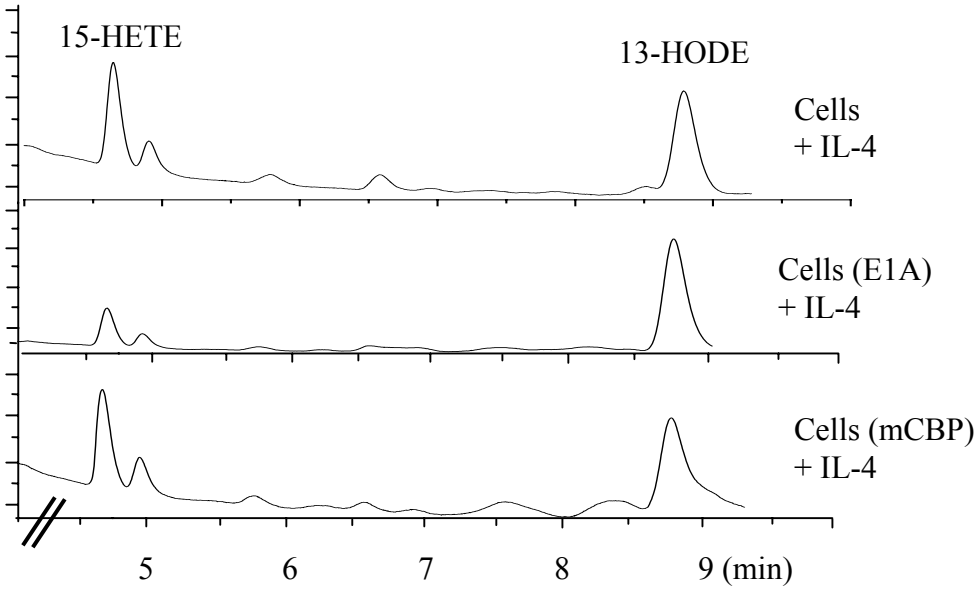


Figure 19b. CBP/p300 was essential for 15-LOX-1 expression. 15 lipoxygenase activity was determined in the cells by HPLC after treatment with IL-4 for 24h. The cell lysates were incubated with AA and lipids were extracted using acidified ethyl acetate. 13-HODE was used as an internal standard.

For the latter experiment, cells were transfected with wtE1A and E1AmCBP vectors and induced with IL-4 for 24 hours. The cell lysate was then incubated with AA, the products were extracted and HPLC was performed. Amount of 15-HETE formed was quantitated with 13-HODE as internal standard. Analysing the 15-LOX activity as a measure for expression of the functional protein, similar amounts of hydroxy fatty acids were observed in control cells and in cells transfected with E1AmCBP (Fig. 19b). In contrast, a significantly reduced 15-LOX-1 activity was observed when the cells were transfected with wtE1A.

4.2 Induction of apoptosis by 15-LOX-1

4.2.1 IL-4 causes apoptosis in A549 cells

15-LOX-1 is a lipid peroxidising enzyme and its expression may cause an increase in the cellular concentration of reactive oxygen species and may lead to cell death. To see if IL-4 stimulation had deleterious effects, A549 cells were examined for apoptosis. TUNEL assay showed that IL-4 (670 pM) induces apoptosis in A549 cells after 72 h of stimulation (Fig. 20a).

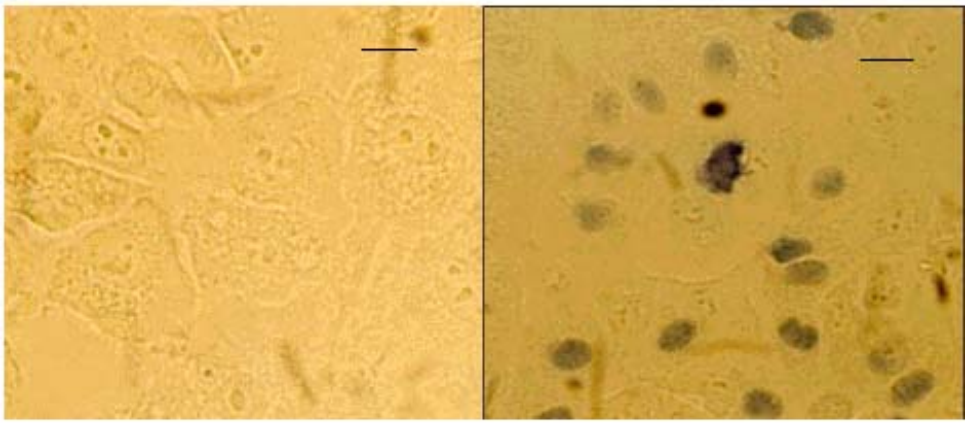


Figure 20a. IL-4 causes apoptosis in A549 cells. TUNEL assay was performed after incubating A549 cells with IL-4 for 72 h. The right panel shows apoptotic cells stained blue. The left panel is the untreated cells.

Annexin V staining showed similar results (fig. 20b). During the early stages of apoptosis, cells lose their phospholipid membrane asymmetry and expose phosphatidylserine at the cell surface while the overall structure of the plasma membrane remains intact. Annexin V binds specifically to phosphatidylserine but cannot penetrate the cell membrane. The exposure of phosphatidylserine on the outside of the cell can be monitored using fluorochrome labelled Annexin V. The green fluorescent staining of the cell indicates apoptosis.

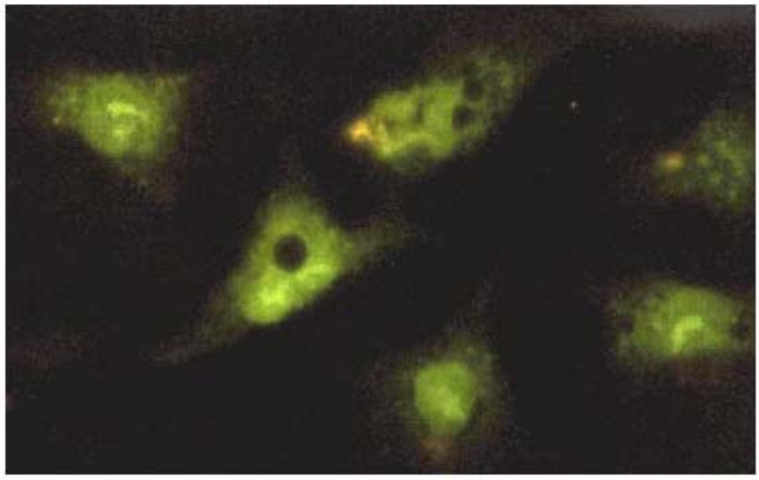


Figure 20b. IL-4 causes apoptosis inA549 cells.. A549 Cells were treated with IL-4 for 72 h and stained with annexin V-fluos. Only the apoptotic cells exhibited fluorescence. The control cells (not treated with IL-4) did not show any fluorescent staining.

4.2.2 IL-4 induces caspase-3 activation via 15-LOX-1

Next was investigated whether or not caspase-3, a downstream caspase involved in the effector phase of apoptosis, is activated when A549 cells were stimulated by IL-4. The activity of caspase-3 using DEVD-pNA as substrate showed an increase upon IL-4 stimuation and thus confirmed the above findings (Fig. 21). NDGA, a general inhibitor of lipoxygenases was used at concentration of 10 µM to verify the effect of 15-LOX-1. A drastic reduction in the caspase-3 activity was measured in NDGA treated cells. Furthermore, the A549 cells were treated with 15-HETE (product of 15-LOX-1). 15-HETE (30 µM) produced a similar increase in caspase-3 activity as IL-4. Treatment of cells with NDGA before 15-HETE incubation did not show any effect on the caspase-3 activity confirming that the product of 15-LOX-1 was required for caspase-3 activation.

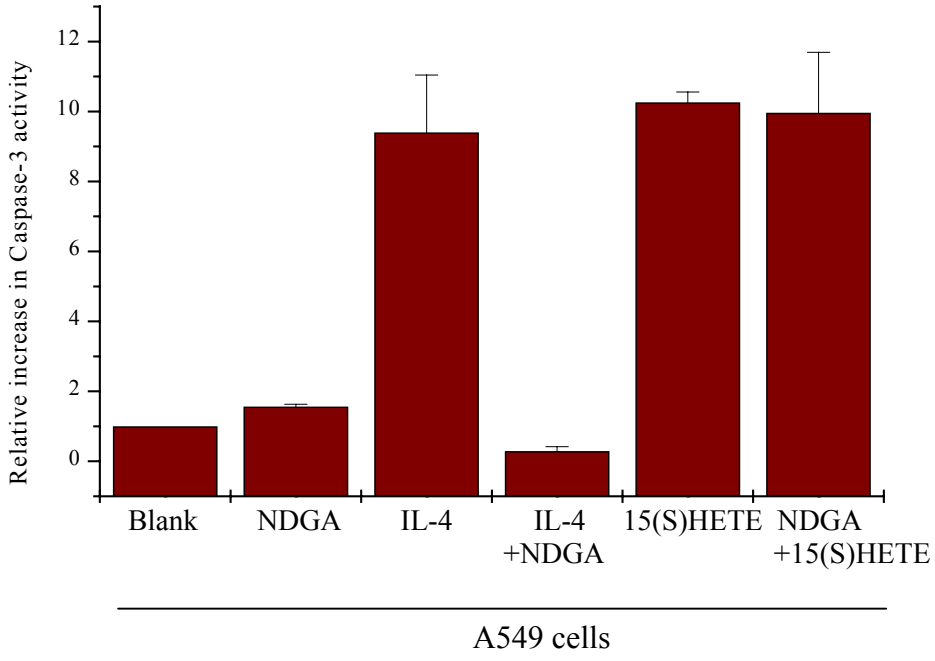


Figure 21. IL-4 induces Caspase-3 activity via 15-LOX-1. Caspase-3 activity was measured in A549 cells after incubation with IL-4 and 15-HETE. NDGA was used as a lipoxygenase inhibitor to assess the effect of 15-LOX-1 in this process. (n = 3).

4.2.3 IL-4 induced apoptosis was mediated by 15-LOX-1

Apoptosis in A549 cells was measured using Cell Death ELISA. This method assays the presence of DNA-histone nucleosomal complexes in the cytoplasm, which is an indicator of

apoptosis. A549 cells were treated with IL-4 for 72 h, cells were treated with 15-HETE or a similar incubation was performed after pre-treatment with NDGA.

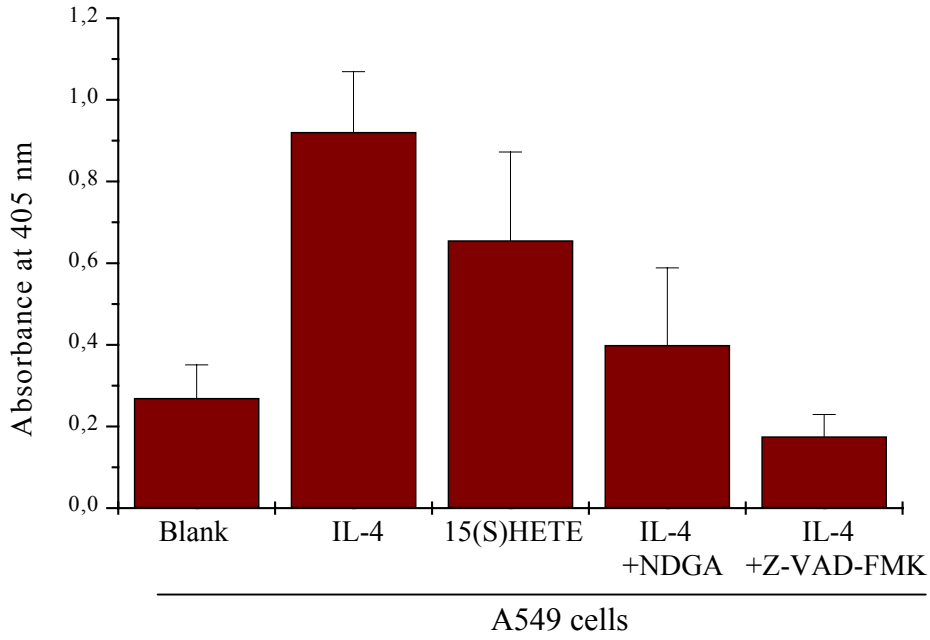


Figure 22. IL-4 induced apoptosis was mediated by 15-HETE and caspase-3. A549 cells were treated with IL-4, 15-HETE or 15-PGJ_2 and amount of apoptosis was measured by Cell Death ELISA. NDGA was used to inhibit the 15-LOX-1 enzyme. (n = 3).

Figure 22 shows the relative increase in the amount of nucleosomal complexes present in the cytoplasm. 15-HETE and IL-4 induced a drastic increase in the amount of apotosis, which was inhibited by NDGA pre-treatment. The cells pre-incubated with a peptide inhibitor of caspase-3, Z-VAD-FMK (100 μM) also showed complete inhibition of apoptosis confirming the central role of caspase-3 in this apoptotic process.

Similar experiments were performed with Beas-2b cells (fig.23), which represent normal tracheobronchial cells. Identical effects were observed in these cells suggesting the process of apoptosis by IL-4 was common in both carcinoma and normal cells.

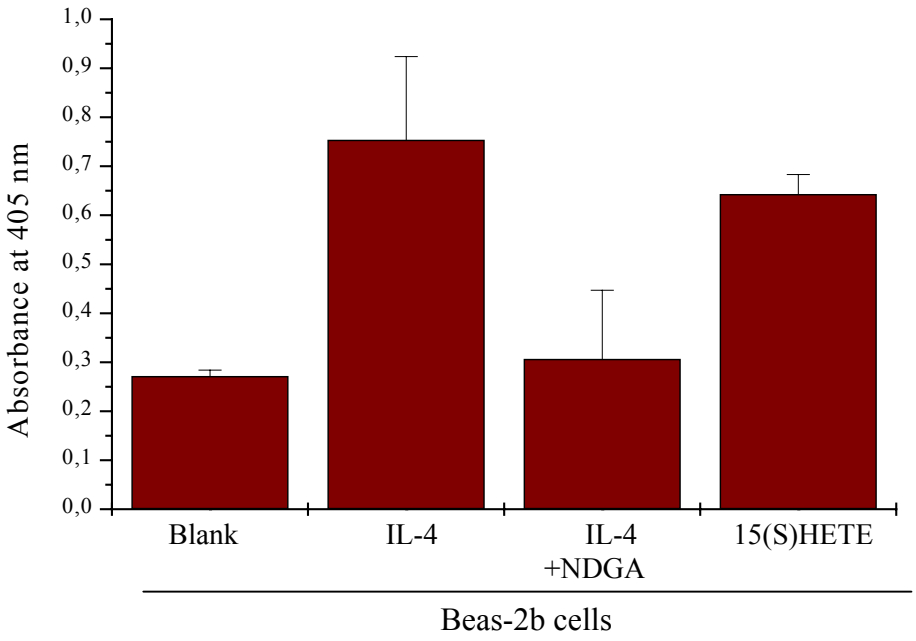


Figure 23. IL-4 causes apoptosis in normal tracheobronchial cells. Normal bronchial cells were treated with IL-4 and the apoptosis was assayed using Cell Death ELISA. (n = 3).

These experiments show the pivotal role of 15-LOX-1 and its product 15-HETE in IL-4 induced apoptosis in lung cells.

4.2.4 15-HETE is a ligand for PPAR γ in IL-4-stimulated A549 cells.

IL-4 treatment of A549 cells results in the upregulation of 15-lipoxygenase, which in turn augmented the production of 15-HETE. 12/15 lipoxygenase products have earlier been shown to act as ligands for PPARγ at relatively high concentrations. To test the hypothesis that 15-HETE could serve as a ligand for PPARγ in this cell system, A549 cells were labelled with [^{14}C]-arachidonic acid and treated with 670 pM of IL-4 for 72 hours. Total protein extracts prepared from the cells were subjected to immunoprecipitation with PPARγ -antibody and protein-A agarose. The lipids were extracted from the immune complex and analysed by

TLC. In IL-4 treated cells alone a radioactive lipid was observed. It was identified as 15-HETE by radio-TLC by co-chromatography of standard 15-HETE (Fig. 24).

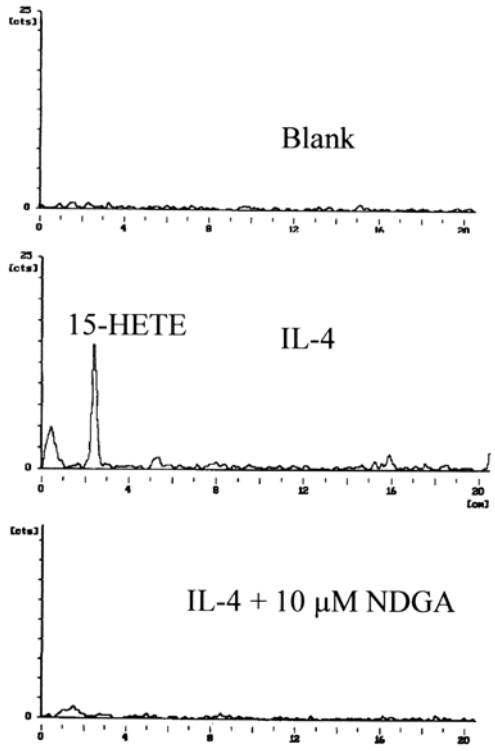


Figure 24. 15-HETE acts as a ligand for PPARγ during IL-4 treatment of A549 cells. A549 cells radioactively labelled with ^{14}C-AA and were treated with IL-4 and the cell lysates were subjected to immunoprecipitation with PPARγ antibody. The lipids were extracted from the immunoprecipitates and subjected to TLC to identify the ligands for PPARγ.

Untreated cells or cells treated with NDGA (10μM) prior to IL-4 challenge failed to show any radioactive ligand for PPARγ. Thus, 15-HETE produced in A549 cells upon IL-4 stimulation acts as a ligand for PPARγ.

4.2.5 IL-4 activates PPARγ via 15-HETE

PPARγ is a nuclear receptor, which upon binding to the ligand gets activated and along with another protein RxR binds to a specified sequence element, PPRE, on the promoter of downstream genes. Binding of the ligand to the receptor is not exclusive to the activation of

the downstream targets of the receptor. Thus, ability of 15-HETE to activate PPARγ downstream genes was tested utilising a PPARγ driven promoter and luciferase reporter.

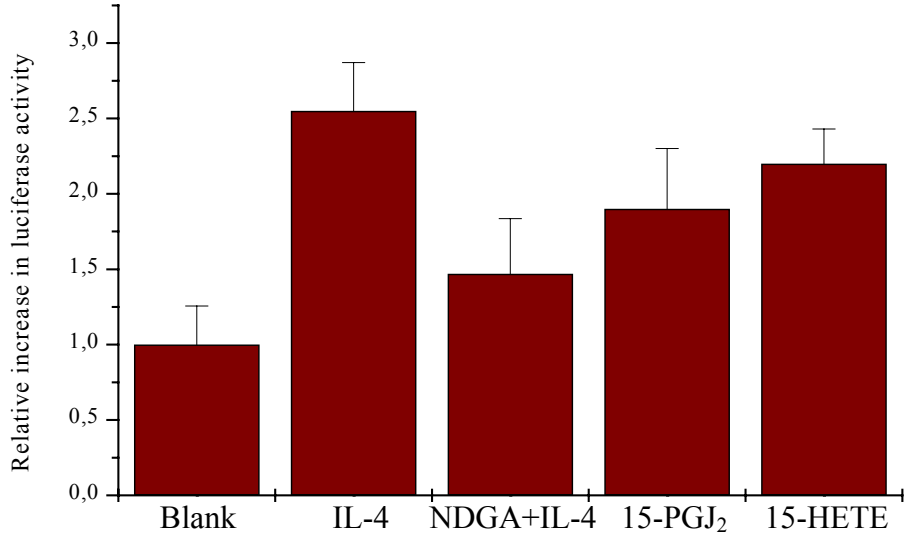


Figure 25. 15-HETE produced in A549 cells upon IL-4 treatment causes an increase in PPARγ dependent promoter activity. PPARγ dependent promoter activation was in A549 cells after transient transfection with a luciferase reporter vector driven by a PPARγ binding element. The transfection efficiency was normalised by co-transfection with a CMV driven β-galactosidase control vector. (n = 3).

IL-4 increased the PPARγ dependent promoter activity (Fig. 25), which was inhibited by 10 μM NDGA indicating that the enzymatic product of 15-LOX-1 was essential for the promoter activation. 15-HETE (30 μM) was also observed to increase PPARγ dependent promoter activity, confirming the functional interaction between 15-HETE and PPARγ receptors. A similar increase in the luciferase activity was observed with 5 μM 15-deoxy-$\Delta^{12,14}$ PGJ_2 (15-PGJ_2), a naturally occurring ligand of PPARγ.

4.2.6 IL-4-induced apoptosis was mediated by PPARγ

Cell Death Detection ELISA detects cytoplasmic DNA-histone complexes indicating apoptosis. IL-4 treated cells showed a significant degree of apoptosis as compared to the untreated controls. 15-HETE (30μM) and 5 μM 15-PGJ_2, a PPAR γ ligand. also caused

equivalent percentage of apoptosis when treated for 72 hours. Cells were transiently transfected with a dominant negative vector for PPARγ and treated with IL-4. In the PPARγ dominant negative vector, PPARγ was leu^{468} was mutated to Ala and Glu^{471} was mutated to Ala (Adams *et al.*, 1997), which prevented the ability to activate transcription. PPARγ dominant negative vector abolished the induction of apoptosis by IL-4 (Fig. 26).

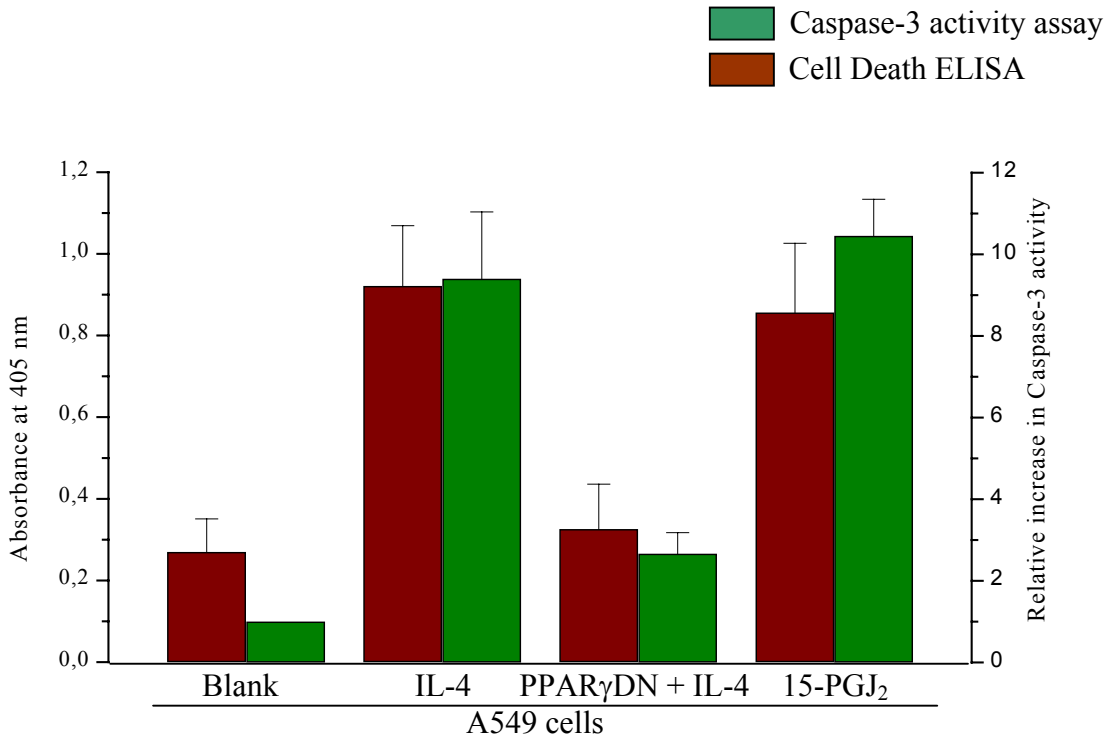


Figure 26. IL-4 induced apoptosis via PPARγ. A549 cells were transiently transfected with PPARγ dominant negative vector (PPARγDN) and treated with IL-4. Apoptosis was measured by Cell Death ELISA and caspase-3 activity assays. (n = 4).

Upon treatment of dominant negative cells with 15-PGJ$_2$ no signs of apoptosis were observed. This indicated that IL-4 induced apoptosis occurs via PPARγ. Similar results were observed with caspase-3 activation too (fig. 26).

4.2.7 PPARγ upregulates cleavage of caspase-8

Caspase-8 is one of the important upstream factors involved in the upregulation of caspase-3 activity. Caspase-8 exists as an inactive 54 kDa molecule which is autocleaved into active p41/42 and p18 molecules. A549 cells treated with IL-4 showed significantly higher levels of the cleaved products as compared to the untreated cells and cells treated with 10 μM NDGA

prior to IL-4 induction (Fig. 27). Similar upregulation of cleavage of caspase-8 was observed with 5 μM of 15-PGJ_2 and 30 μM 15-HETE.

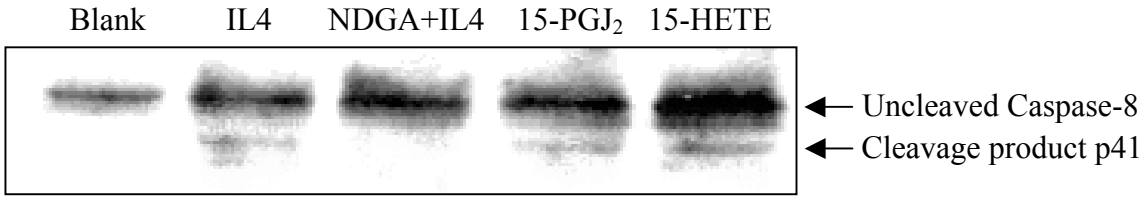


Figure 27. IL-4 causes cleavage of caspase-8 in A549 cells. A549 cells were treated with either IL-4, 15-PGJ_2 or 15-HETE. The cell lysates were analysed by Western blotting for the cleavage of caspase-8. The major cleavage product is 41 kDa while the minor product is 20 Kda, which was too faint to be detected with this antibody.

Caspase-8 cleavage was totally abolished in A549 cells transiently transfected with PPARγ dominant negative vector further emphasising the previous observation (Fig. 28).

4.2.8 IL-4 apoptosis involves death domain receptor pathway

Death domain receptors are family of cell receptors which regulate the survival of the cell in response to various factors such as Fas ligand, TNF-α and TRAIL. These receptors upon activation utilise special adapter proteins to activate the caspase cascades. The involvement of death domain receptors in IL-4 and PPARγ induced apoptosis in A549 cells was verified using a dominant negative vector of FADD, a vital adapter protein in the signaling cascade. This mutant lacks the death effector domain, thereby is unable to transmit the signal.

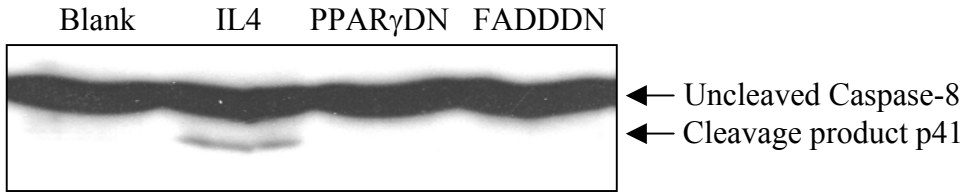


Figure 28. IL-4 induced cleavage of caspase-8 involved death domain receptors. Cells were transiently transfected with the dominant negative vectors of either PPARg or FADD. The IL-4 stimulated cell lysates were analysed by Western blotting for the cleavage of caspase-8.

As shown in Fig. 28, IL-4 induced cleavage of caspase-8 was completely abolished in cells transfected with FADD dominant negative plasmid, demonstrating the involvement of death domain receptors in IL-4 and PPARγ induced apoptosis in A549 cells.

4.2.9 Bid cleavage was not induced by caspase-8

The activated caspase-8 can stimulate apoptosis via either direct cleavage and activation of caspase-3 or the mitochondrial route involving the cleavage of Bid and release of cytochrome c into the cytoplasm.

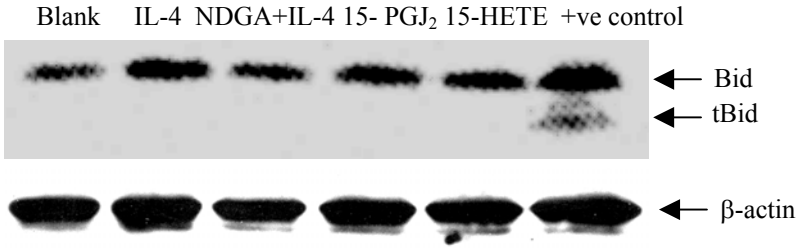


Figure 29. Cleavage of Bid does not occur during IL-4 induced apoptosis. No cleavage of Bid was observed in IL-4 treated A549 cells indicating the absence of the mitochondrial pathway. Jurkat cells treated for 4 h with 25 μM etoposide were used as positive control.

IL-4, 15-HETE and 15-PGJ_2 stimulation failed to induce cleavage of Bid as analysed by western blotting (Fig. 29). This suggests that the caspase-8 directly activates caspase-3 upon IL-4 treatment. As described for type-I cells undergoing apoptosis (Scaffidi *et al.*, 1998).

4.2.10 IL-4 treatment causes activation of Bax and downregulates Bcl-X_L

Bcl-X_L is an antiapoptotic member of the Bcl-2 family. The anti-apoptotic members of the Bcl-2 family of proteins reside in the outer membrane of the mitochondria and prevent the release of cytochrome c. In the cytoplasm, cytochrome c binds to Apaf1 leading via caspase-9 to the activation of caspase-3.

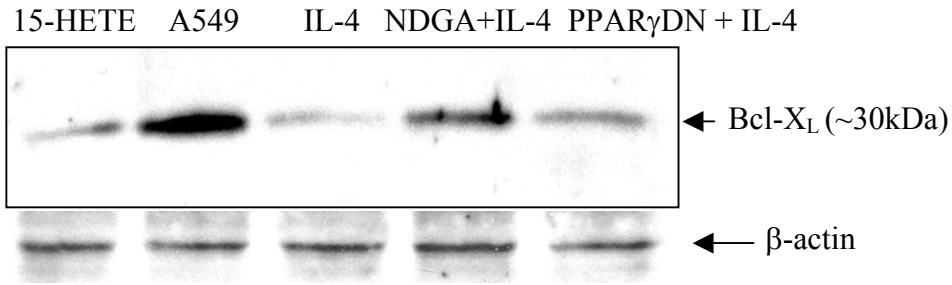


Figure 30. IL-4 causes downregulation of Bcl-X_L in A549 cells. Incubation of A549 cells with IL-4 causes downregulation of Bcl-X_L protein, which was reversed by the treatment of cells with NDGA or by the transient transfection of PPARγ dominant negative vector.

Cells treated with IL-4 showed a marked decrease in the level of Bcl-X_L which was completely reversed by the pre-treatment of the cells with NDGA (Fig. 30) and also by the

transient transfection of PPARγ dominant negative vector. The effect of IL-4 and PPARγ ligands on other members of the Bcl-2 family was investigated. In untreated cells, Bax, a pro-apoptotic member of the Bcl-2 family, was present mostly in the cytoplasm and upon IL-4 treatment a large amount was translocated to the mitochondria (Fig. 31). Porin VDAC, a mitochondrial membrane protein was used as a control to show the purity of the mitochondrial and cytoplasmic preparations.

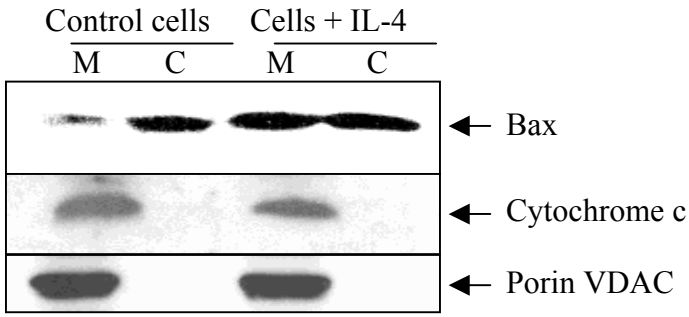


Figure 31. Translocation of Bax to mitochondria. Translocation of Bax to mitochondria from the cytoplasm was observed upon IL-4 treatment of A549 cells. However, no cytochrome c release was observed from the mitochondria upon IL-4 stimulation. Porin VDAC was used as a control to check the purity of the mitochondrial preparations.

The decrease in the Bcl-X_L levels with the concomitant activation of Bax indicates the involvement of the mitochondria in the apoptosis. Regulation by Bcl-2 family of proteins of the efflux of cytochrome c occurs by increasing membrane permeability and alteration of the inner mitochondrial membrane potential. The released cytochrome c interacts with Apaf-1 in the cytoplasm and eventually causes the activation of caspase-3. However, no cytochrome c release was observed upon IL-4 treatment. This implies a cytochrome *c*-independent functional interplay between the pro- and anti-apoptotic members of the Bcl-2 family in IL-4-induced apoptosis in A549 cells.

4.3 12/15-Lipoxygenase exhibits hepoxilin synthase activity

4.3.1 Rinm5F cells express hepoxilin synthase activity

Cultured Rinm5F cells were incubated with AA for 20 minutes and the products were analysed as ADAM ester derivatives by HPLC monitoring fluorescence detection. As shown in Fig. 32, a HXA_3 peak was observed, which co-migrated with an authentic standard.

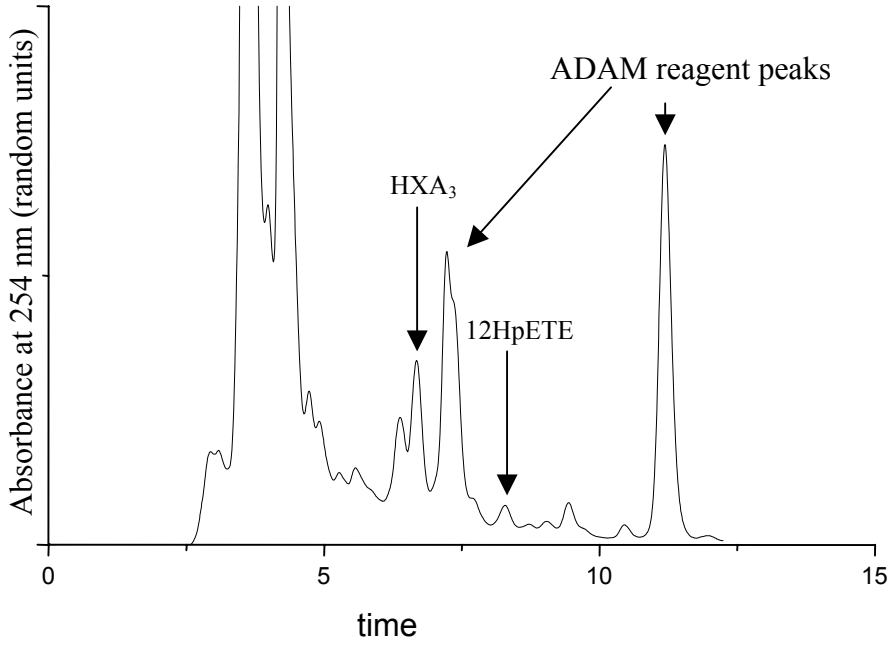


Figure 32. Rinm5F cells synthesise HXA_3 from AA. Rin cells were incubated with AA, lipids were extracted and derivatised with ADAM reagent. The lipids were separated on HPLC, the detection was performed at 254 nm. Marked peaks were ADAM reagent degradation products and do not interfere with the lipid peaks.

Upon longer incubation, e.g. 40 min., HXA_3 peak was substantially reduced and a more polar peak appeared (not shown), which has earlier been characterised as the hydrolysis product 8(S/R),11(R),12(S)-trihydroxy-eicosa-5Z,9E,14Z-trienoic acid and 8(S/R),9(R),12(R)-trihydroxy-eicosa-5Z,8Z,14Z-trienoic acid, trivially known as trioxilins A_3 ($TrXA_3$). As these hepoxilins are hydrolysed by cellular epoxide hydrolases, HXA_3 formation was determined in Rinm5F cells pretreated with 100 µg/ml trichloro-propylene oxide (TCPO), an inhibitor of epoxide hydrolases. After 40 minutes incubation with AA a significant increase in HXA_3 formation was observed (Fig. 33) without appearance of a polar peak.

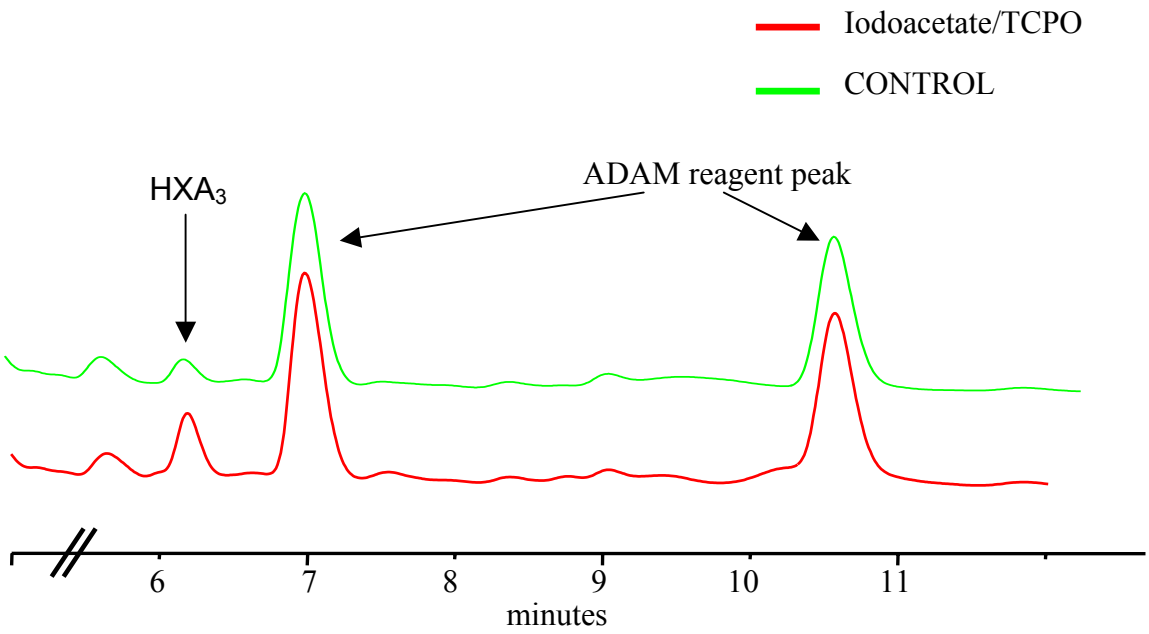
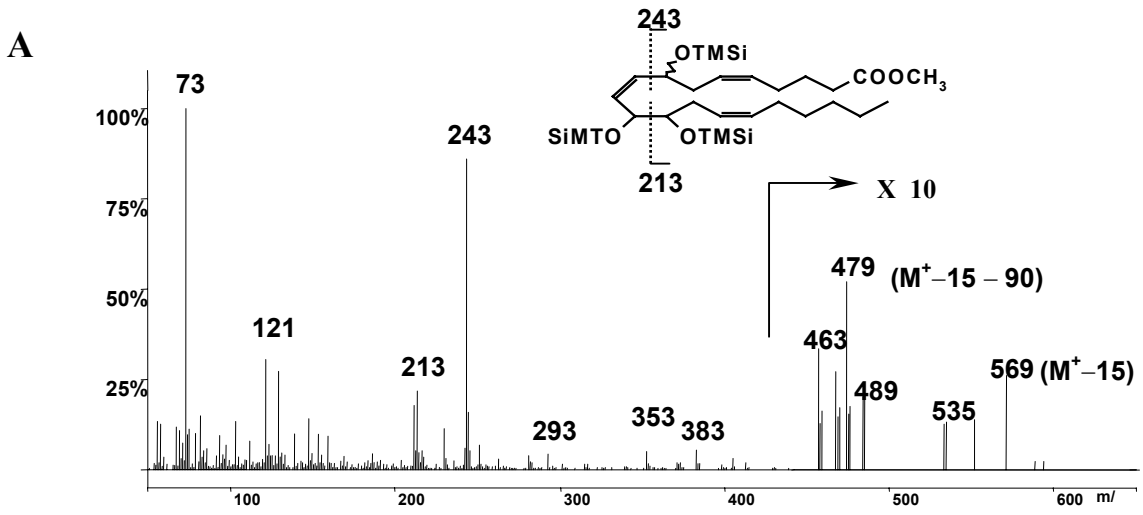


Figure 33. TCPO treatment increases the formation of HXA_3. Rin cells were incubated with AA, lipds were extracted derivatised and separated on HPLC. TCPO, an epoxide synthase inhibitor inhibits the formation of trioxilins, thereby increasing the amount of HXA_3 detected.

To confirm the chemical structure of HXA_3 from Rinm5F cells, the incubation mixture was extracted, the residue acid-hydrolysed (to transform HXA_3 to stable trioxilins) and converted to methyl-silyl derivatives for GC-MS analysis. As shown in Fig. 34B (panel II), a single peak characterising trioxilin A_3 ($TrXA_3$) (Fig. 34A) was observed. No $TrXB_3$ was detected, indicating the absence of HXB_3 formation. Heat-denatured (90°C for 10 min.) cell lysate failed to produce any HXA_3 from 12-HpETE and no 12-HETE from AA, suggesting an enzymatic pathway of formation (Fig. 34B, bottom panel) and the presence of 12-LOX activity was essential in this process.



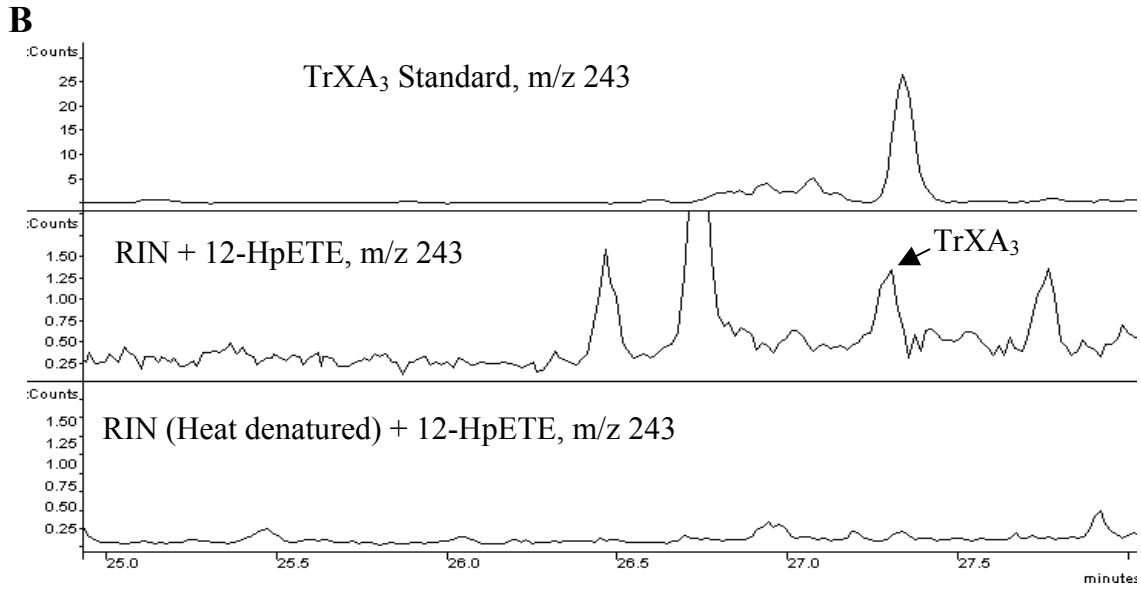


Figure 34. Formation of HXA_3 in Rin cells. Rin cells were incubated with 12-HpETE and the extracted lipids were derivatised, separated and identified by GCMS.
Panel A: Mass spectra of $TrXA_3$ and the fragmentation pattern.
Panel B: Representative mass chromatogram (m/z 243) of Rin cells and heat denatured Rin cells reaction with 12-HpETE.

4.3.2 Glutathione peroxidases inhibit the formation of HXA_3

Recently, the prominent role of PHGPx as a 12(S)-HPETE reductase has been demonstrated in human platelets (Sutherland *et al.*, 2001). In absence of cGPx the reduction of 12(S)-HPETE to 12(S)-HETE was taken over by PHGPx. But, inactivation of both selenoenzymes cGPx and PHGPx by the depletion of glutathione, led to accumulation of 12(S)-HPETE, the metabolism of which was consequently diverted to HXA_3 and HXB_3 formation. Since Rinm5F cells are almost devoid of cGPx as well as PHGPx (Lortz *et al.*, 2000), the formation of hepoxilin was predicted.

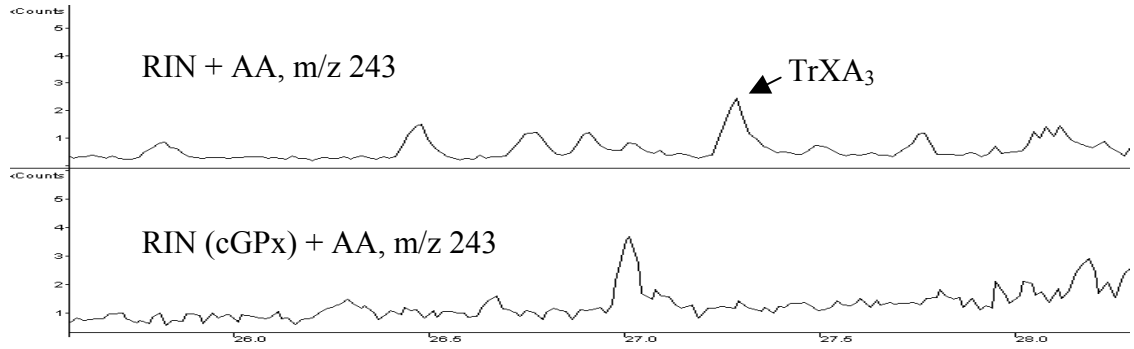


Figure 35. Formation of HXA_3 was regulated by cGPx. Representative mass chromatogram of normal Rin and Rin stably transfected with cGPx and AA. Transfected cells show no production of $TrXA_3$.

As expected these cells synthesised hepoxilin when incubated with AA as substrate (Fig. 35, top panel). Conversely, the presence of GPx should diminish the formation of HXA_3. Indeed, upon incubation with AA cultured RINm5F cells, stably transfected with cGPx did not produce any HXA_3 as determined by GC-MS (Fig. 35, bottom panel). Incubation with 12(S)-HpETE of native or cGPx-transfected RINm5F cells reproduced identical results, to those obtained with AA. The chemical characterisation of the hepoxilin by HPLC and GCMS showed that only HXA_3 and no HXB_3 was formed, which another substantiation of the enzymatic nature of HXA_3 synthesis in Rinm5F cells.

4.3.3 Rat 12/15-LOX exhibits intrinsic HXA_3 synthase activity

Lipohydroperoxidase activity has been attributed to 12/15-Lipoxygenases (Veldink *et al.*, 1997). This activity suggests a role for 12/15-LOX in the synthesis of HXA_3. HeLa cells do not exhibit any intrinsic 12-LOX activity, so these cells were utilised for transfection with rat 12/15-LOX. Upon incubation of cultured transfected cells with AA, neither 12(S)-HETE nor HXA_3 was produced, suggesting the complete down-regulation of 12-LOX by selenoenzymes present in the cell (Fig. 36, top panel). However, pretreatment of transfected cells with by 2 mM diethyl maleate (DEM), which depletes cellular glutathione (GSH) and thus inhibits glutathione peroxidases (Imai *et al.*, 1998), caused significant enhancement of HXA_3 synthesis (Fig. 36, bottom panel). As no HXB_3 formation was detected in GSH- and GPx-depleted cells, the specific HXA_3 synthase activity of 12/15-LOX can be observed.

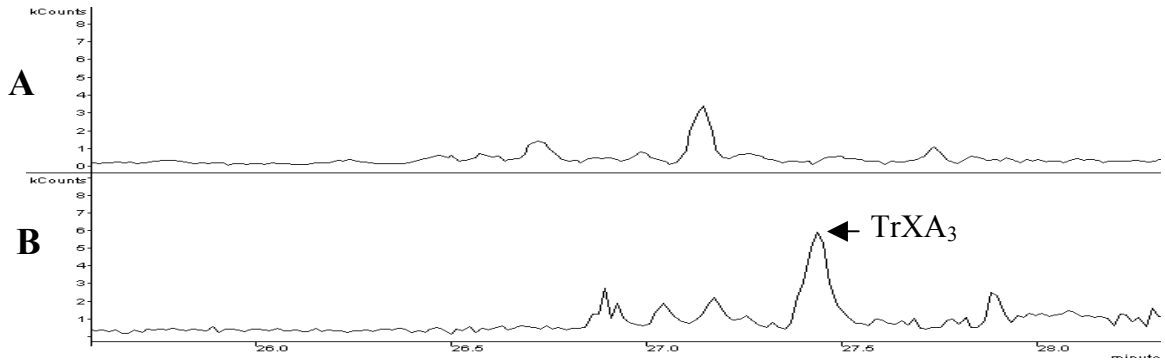


Figure 36. Rat 12/15-LOX exhibits hepoxilin synthase activity. HeLa cells were transfected with 12/15-LOX plasmid and reacted with AA.
Panel A: Hela cells transfected with 12/15-LOX and AA (m/z 243).
Panel B: HeLa cells transfected with 12/15-LOX and treated with DEM and incubated with AA produces $TrXA_3$ (m/z 243). DEM is a glutathione depleting agent.

4.3.4 Hepoxilin synthase activity in Rin cells was depleted by a 12/15-LOX antibody

As seen in the previous experiment, upon 12/15-LOX transfection of HeLa cells hepoxilin synthase activity was observed. To confirm these findings, Rin cell lysates were subjected to immunoprecipitation with 12/15-LOX specific antibody. The lysate, immunoprecipitate and the 12/15-LOX depleted lysate were incubated with 12-HpETE and analysed by GCMS for the production of $TrXA_3$.

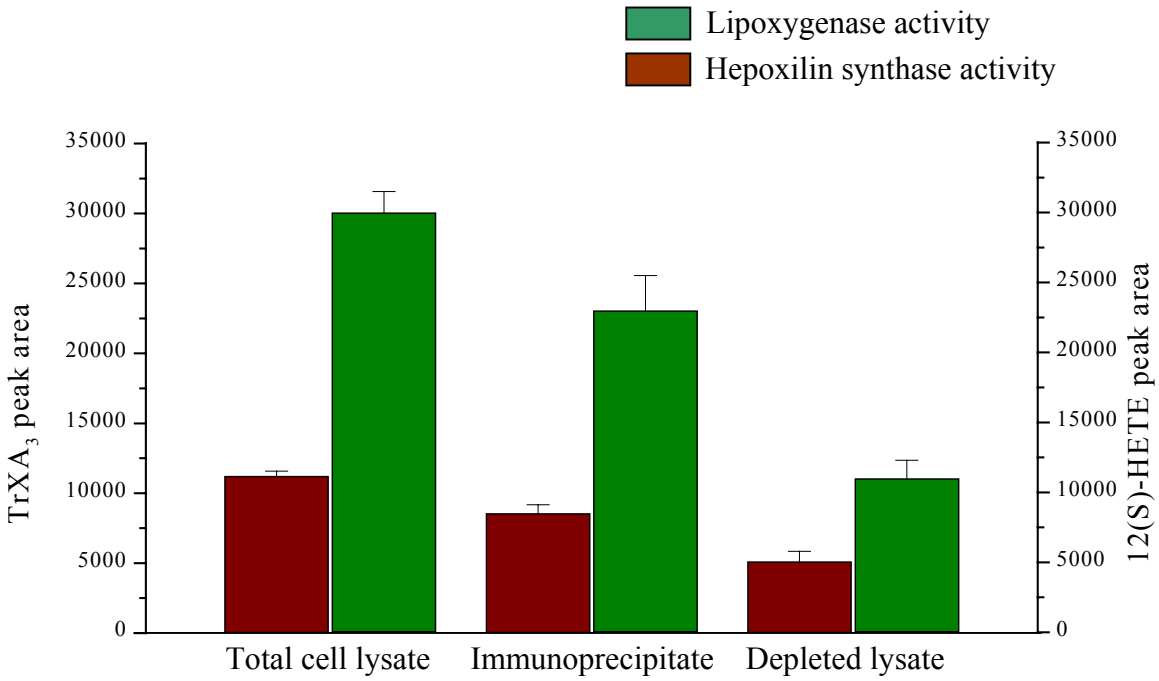


Figure 37: 12/15-LOX antibody depletes hepoxilin synthase activity from RIN cells. Rin cell lysate was subjected to immunprecipitation with 12/15-LOX antibody. The total lysate, immunoprecipitate and the depleted lysate were incubated with 12-HpETE and the production of $TrXA_3$ was measured by GCMS. Similarly lipoxygenase activity was determined by incubating the lysates with AA and analysing with HPLC. 13-HODE was used as internal standard for quantification of both $TrXA_3$ and 12-HETE. (n = 3).

The immunoprecipitated protein shows high amount of hepoxilin synthase activity (fig. 37). There was a significant reduction in the hepoxilin synthase activity observed in the Rin cell lysate after immunoprecipitaion. Since the amount of protein was higher than the amount of antibody complete depletion was not observed. Simultaneously the lipoxygenase activity was also determined by the incubation of lysates with AA and measuring the formation of 12-HETE produced by HPLC. The depletion in the lipoxygenase activity paralleled the hepoxilin synthase activity. 13-HODE was used as an internal standard to normalise the extraction process and for quantification. Nevertheless, these observations confirm the hypothesis that 12/15-LOX was responsible for the hepoxilin production.

4.3.5 Recombinant 12/15-LOX protein exhibits hepoxilin synthase activity

The full length 12/15-LOX gene was amplified from mRNA prepared from RIN cells. The PCR product was cloned into pET15b bacterial expression vector. E.coli BL21 DE3 cells were transformed with this vector and the recombinant protein synthesis was induced by 1 mM IPTG for 3 hours.

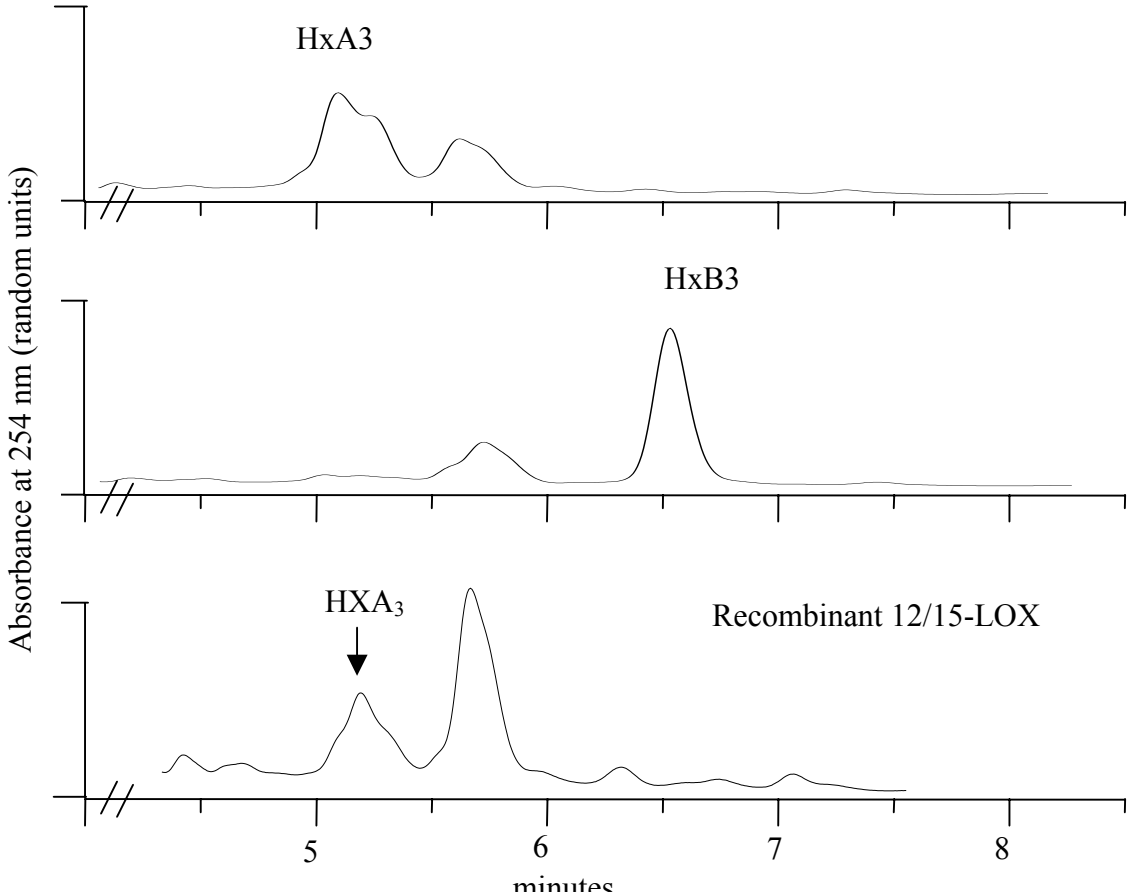
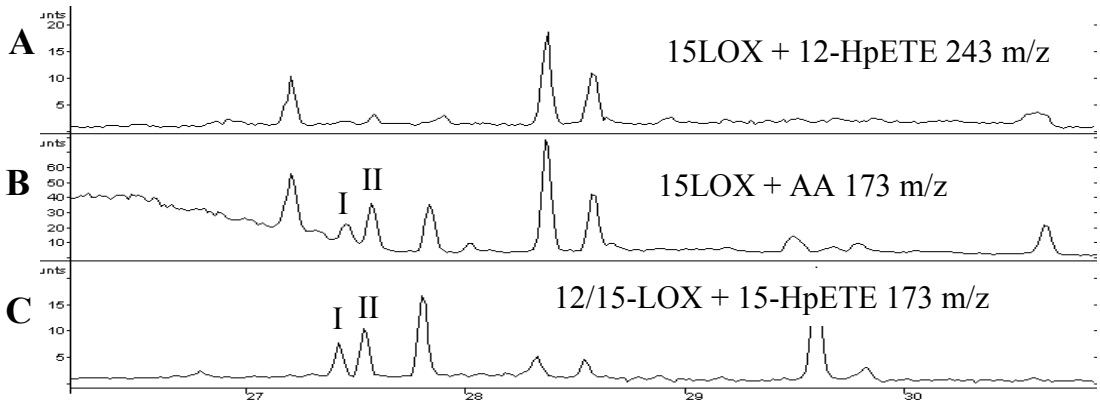


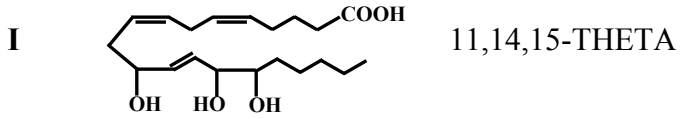
Figure 38: Recombinant 12/15-LOX exhibits hepoxilin synthase activity. Recombinant 12/15-LOX was expressed in bacteria. The bacterial lysate was reacted with 12-HpETE. The extracted lipids were derivatised with ADAM reagent and separated by HPLC. The recombinant enzyme produces a clear HXA_3 peak.

The cell lysate was incubated with 12-HpETE and the extracted lipids were subjected to HPLC. A distinct HXA_3 peak was observed (fig. 38). This experiment confirms the hepoxilin synthase activity of 12/15-LOX.

4.3.6 12/15-LOX produces epoxyhydroxy compounds with 15(S)-HpETE

To test whether the lipohydroperoxidase activity of 12/15-LOX was applicable to other substrates, 15(S)-HpETE was reacted with the recombinant enzyme. The GCMS chromatogram shows the production of two compounds which were identified as 11,12,15-trihydroxyeicosatrienoic acid and 11,14,15-trihydroxyeicotrienoic acid (THETA) (fig. 39). Identical compounds were identified in reaction of rabbit 15-LOX and AA (Pfister *et al.*, 1998). Furthermore, 15-LOX did not produce any epoxyhydroxy compounds with 12-HpETE. Thus, rabbit 15-LOX appears to use only 15-HpETE as substrate while rat 12/15-LOX can accommodate a larger variety of substrates. This unexpected substrate specificity could be due the larger predicted active site volume in the 12/15-LOX as compared to the rabbit 15-LOX.





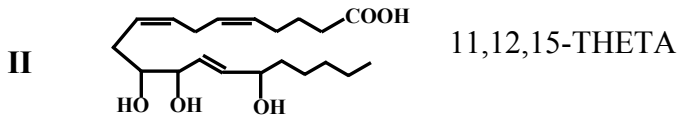


Figure 39: Rat 12/15-LOX produces epoxyhydroxy compounds from substrates other than 12-HpETE.
Panel A: Representative mass chromatogram (m/z 243) for the reaction between recombinant rabbit 15-LOX and 12-HpETE. No $TrXA_3$ was detected. **Panel B:** Chromatogram (m/z 173) of 15-LOX and AA. **Panel C:** Chromatogram (m/z 173) of rat 12/15-LOX with 15-HpETE. In panel B and C formation of compound I and II were observed. The fragmentation profile of the two compounds allowed their identification as **I : 11,14,15-THETA and II: 11,12,15-THETA.**

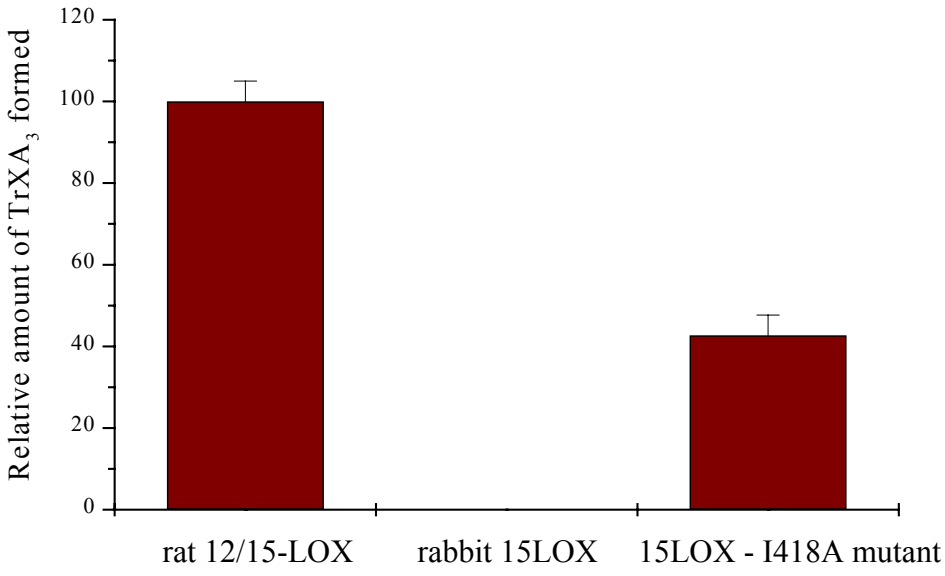


Figure 40. Positional determinant of 12-Lipoxygenation reaction is important for the hepoxilin synthase activity. Recombinant rat 12/15-LOX, wild type rabbit 15-LOX and I418A mutant of 15-LOX were incubated with 12-HpETE and the products were analysed by GCMS. TrXA3 formation was quantified by comparison to the internal standard (15-HETE). (n = 3).

The mutation of isoleucine 418 to alanine (I418A) in rabbit 15-LOX was observed to shift the positional specificity of this towards 12-lipoxygenation (Sloane *et al.*, 1991). Formation of $TrXA_3$ was measured with recombinant rat 12/15-LOX, wild type rabbit 15-LOX and I418A mutant of 15-LOX. As observed before (fig. 39) wild type 15-LOX did not produce any $TrXA_3$ with 12-HpETE, however, the I418A mutant showed a substantial increase in the formation of $TrXA_3$ (fig. 40). This demonstrates the importance of the positional specificity of the 12-lipoxygenase reaction for the hepoxilin synthase activity. These data attest the hypothesis that 12/15-LOX possess a hepoxilin synthase activity and can accommodate different substrates like 12-HpETE and 15-HpETE.

5. Discussion

5.1 Upregulation of 15-LOX-1 expression by IL-4 in lung epithelial cells

Acetylation of proteins is a common principle to modify their biological activity. It impacts protein chemical properties and, thus may alter protein-protein interaction, DNA recognition and protein stability. Histones were the first proteins that have been identified as targets for protein acetylation. Although there are several lines of experimental evidence suggesting the importance of histone acetylation in the transcription of a variety of genes (Grunstein, 1997), its precise role in nucleosome remodelling is still elusive. Several families of histone acetyltransferases (PCAF/GCN5, CBP/p300, TAF(II)250, SRC-1, MOZ) have been characterized in the past and, recently even non-histone nucleic acid binding proteins, such as HMG-1, p53, GATA1 have been identified as acetylation substrates (Sterner *et al.*, 1979; Gu and Roeder, 1997). The consequence of acetylation of these regulatory proteins depends on the internal sites of acetylation. For instance, HMG-1 is acetylated at its DNA binding site, which results in the disruption of its DNA-binding capabilities (Sterner *et al.*, 1979). In contrast, other transcription factors such as p53, GATA1, E2F1 are acetylated outside their DNA binding site and this results in stimulation of DNA-binding (Gu and Roeder, 1997). Sequence alignments have indicated that STAT6 contains several potential acetylation sites and its acetylation has already been reported before (McDonald and Reich, 1999). However, this process was independent of IL-4. The data presented in this study clearly indicate that IL-4 induced transcription of the 15-LOX-1 gene requires upregulation of STAT6 acetylation, which is mainly due to activation of the acetyltransferase activity of CBP/p300. It should, however, be stressed that the acetylation degree of cellular proteins is a resultant of acetylating and deacetylating processes. Thus, an increase in the acetylation degree of STAT6 can either be achieved by activation of acetyltranferases (CBP/p300) and/or by inhibition of deacetylases. Deacetylases have been shown to occur in the nucleus and appear to play an important role in transcriptional repression (Downes *et al.*, 2000). Whether they are recruited by nuclear hormone receptors bound to certain nuclear corepressors is not clear (Ordentlich *et al.*, 2000). It was found that sodium butyrate (non-specific inhibitor of cellular HDAC) alone is capable of inducing 15-LOX-1 expression in A549 cells suggest that transcriptional repression of the 15-LOX gene in resting cells may be due to a preponderance of deacetylating processes over acetyltransferases. It would be of particular interest to elucidate whether it is a general principle for transcriptional repression of 15-LOX-1 gene in mammalian cells. Recently, Kamitani *et al.*, 2001, have observed that treatment of colorectal cell line, Caco-2 with sodium butyrate and other histone deacetylase inhibitors causes an

upregulation of 15 LOX-1 expression and found that this upregulation is linked to the degree of histone acetylation. Recently, the expression of IL-8 gene by lung epithelial cells upon challenge by particulate matter was also shown to be upregulated by histone acetylation (Gilmour *et al.*, 2003). Moreover, the question of whether similar mechanisms may be involved in transcriptional activation of other IL-4-inducible genes remains to be investigated in the future.

In A549 cells the expression of the 15-LOX-1 gene may be inhibited under resting conditions because non-acetylated histones formed by HDAC are bound to the 15 LOX-1 promoter. This mode of transcriptional repression has been reported for a variety of inducible genes and appears to be well characterised (Grunstein, 1997). Histone binding to genomic DNA forms a condensed nucleosomal structure and there is no possibility for the binding of specific transcription factors. Acetylation may induce conformational changes of the histone octamer, which then may provide access to transcription factors for binding to the promoter of relevant genes. Originally, it was postulated that phosphorylation of STAT6 would be sufficient to allow its binding to the 15-LOX-1 promoter. However, the present data indicates that this may not be true. In A549 cells IL-4 induces STAT6 acteylation in addition to phosphorylation and both reactions appear to be required for translational activation of the 15-LOX-1 gene. This conclusion may be drawn form the following experimental data: i) IL-4 increases the activity of cellular acetyltransferases (Fig. 9), particularly of CBP/p300, which are capable of acetylating STAT6 (Fig. 17). ii). The viral oncoprotein wtE1A, an inhibitor of acetyltransferase activity of CBP/p300, prevented STAT6 acetylation, and expression of the functional enzyme. In contrast, its non-inhibitory mutant E1AmCBP was unable to do so (Fig. 17,18,19). iii) Inhibition of STAT6 acetylation by wtE1A prevented STAT6 binding to the 15-LOX-1 promoter (Fig. 18). In contrast, the E1AmCBP mutant did not inhibit the CBP/p300 acetylase activity and also did not prevent STAT6 binding. iv) The histone deacetylase inhibitor sodium butyrate synergistically induced the IL-4-stimulated 15-LOX-1 expression.

Since further acetylation of STAT6 by CBP/p300 acetyltransferases takes place inside the nucleus, tyrosine phosphorylated STAT6, which is inevitably required for homodimerization and subsequent nuclear translocation, becomes an essential precursor. This is strongly supported by the inhibition of STAT6 acetylation by genistein (Fig. 11 and 12). No effect of wtE1A oncoprotein on the tyrosine phosphorylation of STAT6 was observed as checked with anti-phospho-STAT6 antibody in Western blot.

Phosphorylation of transcription factors is a rapid process and our *in vitro* binding assays indicated that phosphorylated STAT6 quickly binds to the naked 15-LOX-1 promoter. On the other hand, *in vivo* expression of the 15-LOX-1 mRNA in intact A459 cells requires at least 11 h and similar observations have been reported for other cytokines (Roy and Cathcart, 1998). This obvious discrepancy together with the results presented in Fig. 12 indicated that *in vivo* the binding of phosphorylated STAT6 to the 15-LOX-1 promoter appears to be inhibited during the first 11 h of IL-4 treatment. Although the detailed mechanism of the inhibitory processes is still unclear our data suggest that the binding of non-acteylated histones may be involved. Acetylation of histones, which appears to be a delayed process in A549 cells, may overcome this inhibitory process, so that acetylated STAT6 can bind to the 15-LOX-1 promoter. For the time being, the reasons for the delayed acetylation of histones and STAT6 remain obscure. Although the cellular acetyltransferase activity is upregulated within 1 h following IL-4 exposure, acetylated histones and acetylated STAT6 could only be detected after a 9 h incubation period.

In summary, it can be concluded from the data that the mechanism of transcriptional activation of the 15-LOX-1 gene by IL-4 does not follow the conventional activation pathway of IL-4 inducible genes. Acetylation of both histones H3 and STAT6 is essentially required for transcriptional activation of the 15-LOX-1 gene, and this acetylation is mainly due to the acetyltransferases activity of CBP/p300. These observations suggest that acetylation is an additional step required in the normal signal transduction pathway induced by IL-4 in these cells. The implication of acetylation in other IL-4 induced pathways, however, needs to be confirmed.

5.2 Induction of apoptosis by 15-LOX-1

In the present study, it has been shown that 15(S)-HETE is bound as a ligand to PPARγ transcription factors (Fig. 24) and is an effector of apoptosis (Figs.20-23). Moreover, treatment of cells with NDGA, a 15-lipoxygenase inhibitor, prevented the PPARγ activation and apoptosis. Identical results were also obtained with PPARγ ligand 15-PGJ_2. To further substantiate the crucial role of 15(S)-HETE for apoptosis *via* PPARγ transcription factor, dominant negative form of PPARγ was used, in which two amino acids (L468A and E471A) have been mutated, thus impairing transcriptional activation and co-factor recruitment (Adams *et al.*, 1997). Transfection of PPARγ dominant negative in A549 cells strongly inhibited the IL-4-induced and 15-PGJ_2-induced apoptosis (Fig. 26), supporting the prominent

role of 15(S)-HETE and 15-PGJ_2 as effectors of apoptosis *via* PPARγ pathway. It must be mentioned that the concentrations of 15-HETE (30 μM) and 15-PGJ_2 (5 μM) used were far higher than the physiological concentrations in the cell. However, this difference should not totally discredit the role of the compounds in the apoptotic process as inside the cell the local concentrations could attain such values. Experiments with normal bronchial epithelial cells BEAS-2B (fig. 23) confirmed the observations in A549 cells and thus underlined the importance of these observations in human allergic inflammatory reactions.

15-LOX-1 is a lipid peroxidising enzyme and has the capability of damaging lipid membranes especially the organelle membranes as seen during the maturation of erythrocytes. This entails an important role for this enzyme in the process of cell death either through apoptosis or necrosis. However, not all LOX expressing cells undergo apoptosis and there is apoptosis in the absence of 12/15-LOX. One of the factors with an regulatory role may be PHGPx. The ability of PHGPx to scavenge the lipid hydroperoxides may play an important role in limiting the damage caused by lipoxygenase. PHGPx has been shown to play an important role in inhibiting free radical induced damage of the mitochondria and consequently apoptosis (Imai *et al.*, 1996; Nomura *et al.*, 1999). IL-4 treatment of A549 cells causes a simultaneous increase in the lipoxygenase activity and a decrease in the PHGPx activity (Schnurr *et al.*, 1996). This leads to a condition of high oxidative stress and may result in cell death. The reduction of the defensive capability of PHGPx could be the decisive step. However, there was no cytochrome c release observed upon IL-4 stimulation (fig. 31) indicating a lack of mitchondrial membrane leakage and damage. Cytochrome c release is a very rapid process, often occurring within the first 30 minutes of the apoptotic process (Lum et al., 2003). Thus the release of cytochrome c may have been finished by the time the assay was performed. The lipoxygenases constitute only one of a number of pathways operative in the death of a cell. A number of other factors, perhaps activated by PPARγ could play a role in the apoptosis observed. Thus, the importance of 15-LOX in the apoptotic process needs to be investigated in detail.

The exact downstream processes of PPARγ activation are still unclear. Involvement of death domain receptor in IL-4-induced apoptosis has been observed. The application of FADD dominant negative vector (Hofmann *et al.*, 2001), lacking the death effector domain, abrogated the apoptotic signal induced by IL-4 or PPARγ (Fig. 28). The involvement of death domain receptors in IL-4-induced apoptosis can be observed by the cleavage of caspase-8 to active subunits p41/42 and p18 (Fig. 27,28) This cleavage and activation is inhibited by

NDGA and PPARγ dominant negative vector demonstrating the vital importance of 15-LOX and PPARγ in the activation of apoptosis signal in this cellular system. Activated caspase-8 has been proposed to stimulate apoptosis through two parallel pathways (Scaffidi *et al.*, 1998). In type-I cells, capsase-8 directly cleaves and activates caspase-3. Type-II cells utilize the mitochondrial pathway through the cleavage of Bid and subsequent release of cytochrome c to amplify the apoptotic signal. Caspase-3, an effector caspase further cleaves PARP and other cellular proteins to cause apoptosis. The IL-4 treated cells do not exhibit Bid activation (Fig. 29), thus suggesting the involvement of the type-I pathway. Earlier, it has been shown that PPARγ promotes the TRAIL induced apoptosis (Ji *et al.*, 2001). TRAIL utilizes various types of death receptors like DR3, DR4 and DR5 to trigger apoptosis (Baker and Reddy, 1998; Ashkenazi and Dixit, 1998). However, it is intriguing to note that IL-4-induced apoptosis in A549 is mediated simultaneously through two different pathways, i.e. through direct activation of caspase-3 and through mitochondrial pathway involving Bax. The activation of Bax and its subsequent translocation to the mitochondria along with the decrease in Bcl-X_L can account for the mitochondrial pathway, however, no cytochrome c release was observed. A number of factors other than cytochrome c, when released from the mitochondria can activate apoptosis (Garl and Rudin, 1998; Matsuyama and Reed, 2000) Thus, the scenario can be explained in the following way: in type I cell death, binding of 15(S)-HETE to PPARγ transcription factor leads to generation of active caspase-8 through activation of FADD protein (Fig 27,28), which subsequently activates downstream effector caspase-3. In IL-4-stimulated cells the binding of 15(S)-HETE to PPARγ transcription factor downregulates Bcl-X_L (Fig. 30,31) and the activation of Bax commits the cell to apoptosis *via* the intrinsic pathway. Bax has also been shown to be involved in a number of apoptotic pathways, especially the DNA damage-induced apoptosis involving p53 (Wu and Deng, 2002) without participation of death receptors. Antidiabetic thiazolidinediones, potent PPARγ agonists, have been observed to induce apoptosis in vascular smooth muscle cells through p53 and Gadd45 pathway, although it is not clear whether PPARγ itself is the effector (Okura *et al.*, 2000). In non small cell lung cancer cells, it has been shown that troglitazone induced DNA damage-inducible Gadd 153 gene. Bax and p53 form an important pathway in DNA damage induced apoptosis (Satoh *et al.*, 2002). Caspase-3 has also been observed to activate the intrinsic apoptotic pathway by the cleavage of anti-apoptotic Bcl-x_L and Bcl-2 to pro-apoptotic components (Cheng *et al.*, 1997; Clem *et al.*, 1998). Thus, caspase-3 activated by other

pathways can activate the mitochondrial route and provide a positive feedback loop for caspase-3 production leading to apoptosis.

In asthma, an upregulation of IL-4 secretion in the blood and higher levels of 15-HETE in the lung and bronchial tissue have been found. It is, therefore, hypothesised that IL-4-induced apoptosis is one of the major causes responsible for the hypertrophy of the bronchial smooth muscle, denuded surface epithelium, thickened basement membrane and infiltration of eosinophils, lymphocytes and mononuclear phagocytes as well as for the apoptotic lesions observed in the lung tissue of asthma patients. It has been reported that PPARγ ligands induce apoptosis in lung cancer cells, and this may be beneficial for the therapy of such cancers (Theocharis *et al.*, 2002; satoh *et al.*, 2002; Inoue *et al.*, 2001; Tsubouchi *et al.*, 2000). In contrast, in chronic inflammatory diseases, such as COPD, the loss of alveolar structures in the lung tissue due to apoptosis may worsen the lung function (Kasahara *et al.*, 2000).

5.3 Biosynthesis of HXA_3 and its regulation by glutathione peroxidases

Rinm5F rat insulinoma cells, which possess leukocyte-type 12-LOX, are devoid of cGPx and PHGPx (Lortz *et al.*, 2000), produced only HXA_3 when incubated with AA or 12(S)-HpETE (Fig. 32,33). Moreover, no HXA_3 was detected in the lysate from heat-inactivated cells (Fig. 34). But, cultured cells stably transfected with cGPx or PHGPx-cDNA did not show any formation of HXA_3 (Fig. 35). These results implied the presence of HXA_3 synthase-like activity in Rinm5F cells and its regulation by glutathione peroxidases. It has been earlier reported that 12/15-LOXs exhibit lipohydroperoxide activity and produce epoxyhydroxy acids from AA (Veldinck *et al.*, 1997; Kühn, 1996; Kühn *et al.*, 1986a). These reports along with other reports on the mechanism of formation of hepoxilin suggested that rat 12/15-LOX could be responsible for the hepoxilin synthase activity. To test the hypothesis recombinant rat 12/15-LOX was overexpressed in bacteria. This enzyme, upon incubation with 12-HpETE or AA, produced HXA_3 (fig. 38). Further confirmation was obtained by performing depletion experiments with 12/15-LOX specific antibodies, which suggest the cohabitation of hepoxilin synthase and lipoxygenase activity in the same protein. Immunoprecipitation with the specific antibody depleted the hepoxilin synthase activity in Rin cell lysates (fig. 37) along with the LOX activity and the activity was recorded in the immunoprecipitated protein. It must be mentioned at this point that immunoprecipitation experiments did not completely deplete the enzyme activity in the lysates. It was of interest to test whether the lipohydroperoxidase activity could be verified using other substrates. 15-HpETE offered similar structural features to test this possibility. Recombinant 12/15-LOX was observed to form two trihydroxy acid

(acid hydrolysed from epoxyhydroxy acids) from 15-HpETE. An earlier work (Pfister *et al.*, 1998) also reported the formation of two trihydroxy acids from AA using rabbit aortic protein extracts. These were identified from their fragmentation patterns as 11,12,15 trihydroxyeicosatrienoic acid (THETA) and 11,14,15-THETA (fig. 39). The fragmentation pattern in the case of 12/15-LOX confirmed that the two THETAs were observed with 15-HpETE as substrate. According to the chemistry of the lipohydroperoxidase reactions, the predicted products of such a reaction with 15-HpETE would have been the THETAs. Further, recombinant rabbit 15-LOX also produced these compounds with AA. However, when 12-HpETE was used as substrate with rabbit 15-LOX, no hepoxilin synthesis was observed. This suggests that the rat 12/15-LOX has a broader range of substrates as compared to the rabbit enzyme. Such a phenomenon could be due the slightly larger predicted volume of the rat 12/15-LOX active site, which would allow the accommodation of different substrates. Furthermore, the shifting of the positional specificity of rabbit 15-LOX to 12-lipoxygenation by I418A mutation caused the formation of hepoxilin (fig. 40). This suggests the importance of the positional determinants of 12-lipoxygenase reaction for the hepoxilin synthase activity. The lipohydroperoxidase activity offers an interesting facet to the enzymatic nature of the lipoxygenases.

HeLa cells overexpressing rat 12/15-LOX did not produce any HXA_3 and 12(S)-HETE under normal conditions due to the presence of abundant glutathione peroxidases. There was, however, a drastic increase in the HXA_3 formation upon treatment with DEM, which depletes cellular GSH and inhibits PHGPx thus, elevating the overall hydroperoxide tone. These data also confirm the hypothesis that the rat 12/15-LOX exhibits an intrinsic HXA_3 synthase activity. The activity was found to be finely regulated by cellular glutathione peroxidases cGPx and PHGPx. This regulation by glutathione peroxidases is in line with earlier observations reported for 5-, 12- and 15-LOX (Weitzel and Wendel, 1993; Sutherland *et al.*, 2001; Schnurr *et al.*, 1996). Normally, in various cell types the presence of glutathione peroxidases exerts the primacy of the reduction pathway over the hepoxilin pathway. However, in experiments with HeLa cells overexpressing the rat 12/15-LOX seemingly selenoenzymes diminished the cellular peroxide tone to such an extent that even a minimum peroxide level essential to trigger the activation of 12-LOX may not be present.

On the basis of these data, it should however be stressed that the mechanism underlying the activation of HXA_3 synthase activity could be primarily due to competition for the same substrate 12-HpETE. Formation of HXA_3 implies the removal of hydroperoxides and thus can be regarded as a counteraction to the permanent oxidative-stressed cellular status. Thus,

synthesis of hepoxilins plays an important role in the processing of cellular hydroperoxides and hence in the overall regulation of the 12-LOX pathway in cells deficient in antioxidant enzymes.

6. PERSPECTIVES

The central observation in the induction of 15-LOX-1 by IL-4 was the delay in the mRNA synthesis. This can be attributed mainly to the kinetics of histone and STAT6 acetylation. The exact mechanisms for this process of gene regulation are however not clear. It would be interesting to study the kinetics of activation of other transcription factors and co-activators and their influence in this process. The expression of 15-LOX-1 is limited to very few cell types. This suggests that repressor mechanisms functioning in other cell types preventing the expression the enzyme. The next focus of research should be directed at elucidating such mechanisms, if at all they exist. This study would be important in understanding the mechanisms underlying the expression of 15-LOX-1 and its function in development.

With the advent of high throughput technology, attempt should be made to study the influence of 12/15-LOXs and its metabolites in various tissues and cell types. Automated cDNA micro-array technology offers such a possibility. This would add to our understanding of the role of these enzymes in the basic processes of the cell and shed light on potential roles in clinical disorders. Transgenic animals overexpressing 12/15-LOX in specific organs and tissues would greatly enhance our knowledge of diseases leading to the development of potential therapeutic strategies.

In this study, the participation of 12/15-LOXs in the apoptotic process in lung cells has been observed. PPARγ, a nuclear receptor plays an important role. Though, it is known that 12/15-LOX metabolites activate this receptor/transcription factor, the downstream targets of the factor are not known. The mechanism leading to activation of the death receptor also remains to be clarified. Antidiabetic thiazolidonenes are known activators of PPARγ and their involvement in the apoptotic process has tremendous therapeutic potential, especially in cancer chemotherapeutics.

Hepoxilins are a novel class of 12/15-LOX metabolites generally observed during the lack of or weakening of the antioxidant defence mechanism in the cell. The nature of influence they could have in the regulation of pro-oxidant enzymes is another interesting area of research. Another interesting aspect of study would be elucidation of the structural aspects of the hepoxilin synthase activity. Site-directed mutagenesis studies could be employed to clarify the mechanism of action.

7. References

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PUBLICATIONS

1. **P. Shankaranarayanan,** S. Nigam (2003) Biosynthesis of hepoxilins: evidence for the presence of a hepoxilin synthase activity in rat insulinoma cells. ***FEBS Lett.*** **538**:107-112.
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4. M. Sutherland, **P. Shankaranarayanan**, T. Schewe and S. Nigam (2001) Evidence for the role of PHGPx in the regulation of the 12-LOX pathway in human platelets. ***Biochem.J***. **353**:91-100.
5. **P. Shankaranarayanan,** M. Sutherland, T. Schewe and S. Nigam (2001) Novel aspects related to biosynthesis and biological functions of hepoxilins. Interrelationship with phospholipid hydroperoxide glutathione peroxidase (PHGPx). ***Advances in Prostaglandin, Thromboxane and Leukotriene Research***. pp. 25-29.

CURRICULUM VITAE

Personal

Name:	SHANKARANARAYANAN PATTABHIRAMAN
Date of Birth:	3rd May 1971
Marital Status:	Single
Address:	Apartment 13 Hindenburgdamm 96A 12203 Berlin, Germany
Phone number:	+49-30-8445 2758
E mail address:	cbpp300@yahoo.com
Nationality:	Indian
Languages:	English, Hindi, Tamil, German

Professional

School:	Senior Secondary School Leaving Examination form the Central Board of Secondary Education, India. P.S. Senior Secondary School, Madras, India (May 1988)
Degree:	Bachelor of Pharmacy (HONOURS) from Birla Institute of Technology and Science, Pilani, India. (August 1988 – June 1992).

Academic positions held

June 1992-October 1994.Research Assistant, Central Drug Research Institute, Lucknow, India

November 1994- February 1999. Project Assistant, Centre for Cellular and Molecular Biology, Hyderabad, India.

March 1999-present. Wissenschaftliche Mitarbeiter, University Clinic Benjamin Franklin, Berlin, Germany.

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